Host immune response to tuberculous meningitis

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Host immune response to tuberculous meningitis

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copromotor: prof.dr. J.F. Schoeman

Voor Sanne

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1 General introduction

INTRODUCTION

Tuberculosis (TB) is still a major global health challenge with a high morbidity and mortality. In 2012, an estimated 8.6 million people developed TB of which 0.5 million were under 15 years of age. 1.3 million people died (8% children) from the disease despite effective anti-tuberculous medication [1]. Although the situation has been improved in many areas around the world, the South African TB burden even deteriorated. With at least 1 in every 100 people developing TB each year, South Africa has together with Swaziland world's highest TB incidence rates [1]. Central nervous system involvement, mostly tuberculous meningitis (TBM), is considered to account for approximately 1% of all cases of TB [2]. Although TB is predominantly a pulmonary disease, extrapulmonary involvement is particularly common in young children and immunocompromised individuals [3]. With an incidence rate of 22%, TBM appeared to be the commonest form of childhood bacterial meningitis in the Western Cape of South Africa [4].

Pathophysiology and clinical presentation of tuberculous meningitis

TB is caused by *Mycobacterium (M.) tuberculosis* and starts with inhalation of infectious droplets, followed by colonization of alveolar macrophages [5]. Interestingly, exposure to *M. tuberculosis* not always leads to active disease, with disease progression being determined by the ability of the host immune system to eradicate or control *M. tuberculosis* [6]. After exposure, there are 3 different clinical outcomes which include, 1) the absence of any clinical or laboratory evidence of infection, 2) infection without clinically active disease, so called latency, and 3) active disease. During disease progression from latency, bacteria may disseminate to local lymph nodes and bloodstream, whereupon spread throughout the systemic circulatory system may occur and can lead to the development of a caseous granuloma in the meninges, adjacent brain tissue, or ventricular ependyma. Rupture of this granuloma into the subarachnoid space causes the clinical features of TBM. In most cases, TBM develops within a few months of the primary infection [7]. Symptoms usually start insidiously with a prodromal period of non-specific symptoms. As the disease progresses, neck stiffness, loss of consciousness, motor paresis and convulsions invariably follow. The diagnosis is often delayed and only considered once irreversible neurological damage has already occurred [8, 9]. Untreated, the condition is almost always fatal with a median time to death of 19.5 days [10]. Even for those treated, TBM is associated with high rates of mortality and morbidity; about 80% of children with advanced disease at diagnosis will suffer severe neurological sequelae [8, 9].

A successful host response to an invading pathogen requires precise coordination of the components of the immune system [11]. The host immune response to

M. tuberculosis relies on both innate and adaptive components. The innate immune system is able to recognize highly conserved motifs on pathogens that are not found on the cells of higher eukaryotes. These motifs are called pathogen-associated molecular patterns (PAMPs) and can bind to pattern recognition receptors (PRRs). Activation of these receptors on antigen-presenting cells induces a signalling cascade by the expression of co-stimulatory molecules and cytokines and consecutive activation of the adaptive immune system [12]. Toll-like receptors (TLRs) are a group of cellular adaptor proteins that play a major role as PRRs in the initiation of innate immune responses. TLR activation results in various functional outcomes, including the secretion of inflammatory cytokines, the regulation of phagocytosis and the induction of host defense mechanisms leading to direct antimicrobial activity but also to local tissue damage [12–14].

Many of the signs, symptoms, and sequelae of TBM result from an immunologically directed inflammatory response to the infection [15]. Alterations in cytokine levels in patients with TBM can affect the function of the immune system and directly impact the course of TBM. In particular, the balance between pro- and anti-inflammatory cytokines may play an important role in disease progression [15]. From previous studies we know that several pro- and anti-inflammatory cytokines (e.g. tumour necrosis factor [TNF]-α, interferon [IFN]-γ, interleukin [IL]-1β, IL-6, IL-8, and IL-10) are upregulated in the cerebrospinal fluid (CSF) of patients with TBM compared with other types of meningitis [16–22]. A disease-specific host inflammatory response to TBM has been suggested but has not been thoroughly investigated [23].

Role of vitamin D in the pathophysiology of TBM

Hypovitaminosis D is a widespread disorder in developing countries [24] and is, in addition to infectious diseases and malnutrition, among the most prevalent childhood health disorders [25]. Vitamin D is a generic term for a group of steroid hormones that are mainly derived from cutaneous photosynthesis in presence of Ultraviolet-B (UVB) radiation. Over the last two decades, low levels of vitamin D have been found to be strongly associated with inflammatory diseases and those of long latency, such as multiple sclerosis, rheumatoid arthritis, diabetes, and *M. tuberculosis* disease [26–28]. Since Rook et al. [29] demonstrated that the active form of vitamin D, 1,25-dihydroxyvitamin D3 (1,25D) could inhibit the replication of *M. tuberculosis* there is growing evidence that vitamin D is involved in the pathophysiology of TB [30]. Activation of monocytes by *M. tuberculosis* through the Toll-like receptor pathway can lead to a vitamin D-dependent production of pro- and anti-inflammatory cytokines and the expression of antimicrobial peptides, such as the cathelicidin LL-37 [31].

Seasonal variation in the incidence of diseases caused by *M. tuberculosis* (including TBM) has been described, but the exact cause of this phenomenon remains unknown [32, 33]. Intensified transmission during winter periods, under conditions of overcrowded, poorly ventilated housing, might play a role [32]. Vitamin D deficiency due to diminished UVB exposure in winter season has also been suggested as contributing factor [26, 33]. Probably the oldest evidence for the association of TBM with low serum levels of vitamin D is described in a dark skinned Homo Erectus fossil found in Turkey [34]. Warm interglacial's probably facilitated the northward migration of early hominins into the temperate regions of Europe and Asia. Investigation on the endocranial surface of the frontal bone showed lesions characteristic for TBM [34]. Nowadays, Non-Western populations who migrate to countries at higher latitudes are still known to be at risk for vitamin D deficiency [35]. Skin type, time spent outdoors, age, low socioeconomic status, wearing of body-covering clothes, and a diet low in fish and dairy products are important contributory factors associated with vitamin D deficiency in immigrants [35]. Whether acculturation, the process that occurs when members of a minority group adopt the cultural patterns of the host country [36, 37] influences migrants' vitamin D status in time is unknown.

Diagnosis and treatment

Early initiation of treatment, and therefore early diagnosis of TBM, is the most critical factor affecting morbidity and mortality [38, 39]. Diagnosis is often delayed due to the non-specific nature of early symptoms such as cough, weight loss, fever, vomiting and lethargy. With disease progression, a more definitive clinical picture becomes manifest [9]. Identification of *M. tuberculosis* in CSF provides bacteriological confirmation of TBM. Due to the paucibacillary nature of TBM, CSF microscopy for acid-fast bacilli (AFB) [40] and CSF culture has low sensitivity (<20% and <50% respectively) [9, 41, 42] compared to clinical criteria. Although culture provides the accepted "gold standard" it has little clinical utility, since it takes up to 42 days to confirm a positive result. Nucleic acid amplification tests (NAATs) have been introduced to provide rapid TB diagnosis and enhanced sensitivity compared to smear microscopy [43–47]. Although primarily developed for the analysis of respiratory specimens, these methods are also used in non-respiratory specimens, like CSF. Among the most promising new methods for diagnosing TB are antigen-detection assays based on the detection of lipoarabinomannan (LAM), a *Mycobacterium*-specific lipopolysaccharide of the bacillus cell wall [48]. Whether LAM detection is of diagnostic value in childhood TBM is unknown.

In TBM, the principles of treatment are still derived from observational studies and clinical practice rather than from controlled trials [49]. Isoniazid and rifampicin are

the key components of the TBM drugs regimen with the perceived need for long-term treatment (9-12 months) to prevent disease relapse [49]. Although treatment regimens with first-line drugs can cure around 90% of TB cases globally, there is an alarming increase in multidrug-resistant (MDR)-TB over the last years [1, 50]. MDR-TB is caused by *M. tuberculosis* resistant to both isoniazid and rifampicin. Extensively drug-resistant (XDR)-TB is additionally resistant to a fluoroquinolone and an injectable second-line anti-TB medication. As drug susceptibility testing (DST) requires a microbiological diagnosis, the diagnosis of MDR-TB in children is often made presumptively based on an MDR-TB source case or first-line treatment found to be failing. MDR-TBM has very poor outcome, [51–54] but there is little data regarding children.

AIMS AND OUTLINE OF THE THESIS

Efforts to control TB are still hindered by gaps in knowledge that exist in diagnosis, prevention and treatment of this disease. Although the clinical presentation and histopathological mechanisms of TBM are well described over the last decades, [49, 55] the cellular and molecular mechanisms are still poorly understood. With this thesis we aim to better understand the host immune response to TBM, explore the role of vitamin D and its related biomarkers in TBM, and improve early diagnosis in order to direct future diagnostic, preventive and therapeutic strategies.

In the first part of this thesis disease specific biomarker patterns in CSF and serum of children with signs and symptoms suggestive of meningitis resident in a TB endemic area will be investigated using multiple statistical analyses (Chapter 2). Next, the influence of sunshine hours on the incidence rate of TBM (Chapter 3) and the role of vitamin D and related biomarkers in the pathophysiology of TBM (Chapter 4) are studied. In the last chapter the association between migration status and serum vitamin D levels in a Dutch paediatric population is investigated (Chapter 5).

In the second part of this thesis two chapters focuses on improvement of early diagnosis of childhood TBM. The first study describes the diagnostic accuracy of an urinary antigen-detection assay based on lipoarabinomannan (LAM) (Chapter 6) and the second study describes the benefit of commercial nucleic acid amplification tests for the accelerated diagnosis of childhood TBM (Chapter 7). In the last chapter, the impact of drug resistance on clinical outcome in children with TBM is investigated (Chapter 8).

The third part contains the general discussion of the thesis with an overview of the studies and future perspectives (Chapter 9) followed by a brief summary in English and Dutch (Chapter 10).

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Part I Host immune response to tuberculous meningitis and the role of vitamin D

2 Host immune response to tuberculous meningitis

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ABSTRACT

Background – Tuberculous meningitis (TBM) is a severe complication of tuberculosis (TB) predominantly affecting young children. Early treatment is vital to prevent morbidity and mortality, emphasising the importance of early diagnosis. The lack of sensitive methods for early diagnosis is the most common cause of delay. Attempts have been made to develop simplified tests for TB but their diagnostic power remains poor. The clinical picture of TBM is mainly driven by the host's immune response to *Mycobacterium tuberculosis*; therefore, identification of disease-specific biomarkers may have diagnostic and therapeutic value and improve our understanding of its pathogenesis.

Methods – We investigated disease-specific biomarkers of childhood TBM in a cohort of children aged 3 months–13 years with symptoms and signs suggestive of meningitis. Cerebrospinal fluid (CSF) and serum from 56 patients with and 55 without TBM were assessed for 28 soluble mediators.

Results – Unsupervised hierarchical clustering analysis revealed a disease-specific pattern of biomarkers for TBM relative to other types of meningitis. A biomarker-based diagnostic prediction model for childhood TBM based on CSF concentrations of interleukin-13 (cut-off value: 37.26 pg/ml), vascular endothelial growth factor (cut-off value: 42.92 pg/ml), and cathelicidin LL-37 (cut-off value: 3221.01 pg/ml) is presented with a sensitivity of 0.52 and a specificity of 0.95.

Conclusions – These data highlight the potential of biosignatures in the host's CSF for diagnostic applications and for improving our understanding of the pathogenesis of TBM to discover strategies to prevent immunopathological sequelae.

INTRODUCTION

A third of the world's population is currently infected with *Mycobacterium (M.) tuberculosis*, and each year > 1.5 million people die from tuberculosis (TB) [1]. Central nervous system (CNS) involvement occurs in ~1% of all cases of TB, among which cases of tuberculous meningitis (TBM) are the most severe, frequently occurring during early childhood [2]. The haematogenous transmission of bacilli from a primary pulmonary focus can lead to the development of a caseous granuloma – termed a Rich focus – in the meninges, adjacent brain tissue, or ventricular ependyma. Rupture of a Rich focus into the subarachnoid space causes the clinical features of TBM. In most cases, TBM develops within a few months of the primary infection [3]. The outcome of TBM is often poor despite adequate anti-TB therapy. Early treatment initiation is the most significant factor affecting morbidity, mortality, and health care costs, emphasising the importance of early diagnosis [4].

Despite the severity of TBM, the cellular and molecular mechanisms underlying its pathophysiology are poorly understood [5]. A successful host response to an invading pathogen requires precise coordination of the components of the immune system [6]. Many of the signs, symptoms, and sequelae of TBM result from an immunologically directed inflammatory response to the infection [7]. Alterations in cytokine levels in patients with TBM can affect the function of the immune system and directly impact the course of TBM. In particular, the balance between pro- and anti-inflammatory cytokines may play an important role in disease progression [7]. Several pro- and anti-inflammatory cytokines (e.g. tumour necrosis factor [TNF]-α, interferon [IFN]-γ, interleukin [IL]-1β, IL-6, IL-8, and IL-10) are upregulated in the CSF of patients with TBM compared with other types of meningitis [5, 8-13]. A disease-specific host inflammatory response to TBM has been suggested but has not been thoroughly investigated [14].

Using emergent statistical techniques, such as unsupervised hierarchical clustering (UHC) analysis, principal component analysis (PCA), and pathway analysis, we investigated the disease-specific biomarker expression profile of childhood TBM. We also evaluated the value of biomarker-based diagnostic prediction models for TBM.

METHODS

Study population and case definition

We conducted a prospective hospital based cohort study of children aged 3 months to 13 years, with symptoms and signs suggestive of meningitis, admitted to Tygerberg Hospital, Cape Town, South Africa between November 2009 and November

2012. The symptoms and signs of meningitis included one or more of the following: headache, irritability, vomiting, fever, neck stiffness, convulsions, focal neurological deficits, altered consciousness, and lethargy. All children with suspected meningitis were classified into the TBM or Non-TBM Groups according to the diagnostic criteria proposed by Marais *et al* [15].

Microbiological assessment of cerebrospinal fluid

In patients with suspected TBM, we performed auramine staining for direct fluorescence microscopy, BACTEC™ MGIT™ 960 Mycobacterial Detection System (Becton Dickinson and Company – Diagnostic Systems, Hunt Valley, MD, USA) culture, and two commercial nucleic-acid amplification tests: the GenoType® MTBDRplus (Hain Life Science GmbH, Nehren, Germany) and Xpert™ MTB/RIF (Cepheid, Sunnyvale, CA, USA) assays. All tests were performed according to the manufacturers' guidelines. Gram staining, Indian ink staining, and blood cultures were undertaken in all cases as part of routine care. A polymerase chain reaction (PCR) assay for viruses, including a herpes virus panel, enterovirus, and mumps virus, was performed when viral meningitis was suspected.

The Tuberculous Meningitis Group

TBM was classified as 'definite' when acid-fast bacilli were evident in, *M. tuberculosis* was cultured from, or *M. tuberculosis* was detected by one of the two commercial nucleic-acid amplification tests in the CSF of a patient with symptoms or signs suggestive of the disease. TBM was classified as 'probable' when patients had a diagnostic score ≥ 12 when cerebral imaging was available and ≥ 10 when imaging was unavailable. TBM was classified as 'possible' when a patient had a diagnostic score of 6–11 when cerebral imaging was available and 6–9 when imaging was unavailable. Diagnostic scores were based on the uniform clinical case definition of Marais *et al* [15].

The Non-Tuberculous Meningitis Group

The Non-TBM Group included bacterial (BM), viral (VM) and No meningitis. BM was identified when either a bacterial pathogen was confirmed by microscopy and/or culture, or the CSF examination showed at least one of: turbid appearance, leucocytosis of >100 cells/μL in isolation or leucocytosis 10-100 cells/μL and either elevated protein >1 g/l or CSF glucose <50% of serum glucose [16]. VM was diagnosed when either a viral pathogen was confirmed by PCR or the clinical outcome was favourable with only supportive and/or antiviral therapy and CSF leucocytes count of ≥ 10×10^6/L and other causes of meningitis were excluded [17, 18]. When an alternative cause was

identified that explained the clinical presentation together with a leucocyte count in the CSF < 5 x10^6/L, a diagnosis of "No meningitis" was made.

Sample preparation

Blood and CSF samples were collected from all children with suspected meningitis during a routine diagnostic workup. All serum and CSF samples were centrifuged, and the supernatant was aliquoted into sterile polypropylene microtubes and stored at –80°C until use. CSF supernatant was passed through a hydrophilic Durapore® polyvinylidene difluoride filter membrane (MultiScreen$_{HTS}$-GV plate 0.22 µm; EMD Millipore Corporation, Billerica, MA, USA).

Multiplex cytokine and chemokine analysis

A panel of 27 host biomarkers in serum and CSF samples were measured by the Luminex multiplex bead array technology (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The panel included IL-1β, IL-1 receptor antagonist (IL-1RA), IL-2, IL-4 – IL-10, IL-12 (p70), IL-13, IL-15, IL-17, IFN-γ, TNF-α, macrophage inflammatory protein (MIP)-1α, MIP-1β, granulocyte colony-stimulating factor (G-CSF), granulocyte–macrophage (GM)-CSF, eotaxin, basic fibroblast growth factor (bFGF), monocyte chemotactic protein (MCP)-1, regulated upon activation normal T-cell expressed and secreted (RANTES), IFN-γ-induced protein (IP)-10, platelet derived growth factor BB, and vascular endothelial growth factor (VEGF). Prior to analysis, serum samples were diluted 1:4 with the sample diluent provided in the kit as recommended by the manufacturer. Assays were read on the Bio-Plex 200 platform and the Bio-Plex Manager software 6.0 was used for bead acquisition and analysis.

Cathelicidin LL-37

The concentration of cathelicidin LL-37 in serum and CSF samples was assessed using an enzyme-linked immunosorbent assay kit (USCN Life Science Inc., Houston, TX, USA). The manufacturer's protocol was followed and serum samples were diluted 1:500 with phosphate buffered saline. Initially, the intra-assay coefficient of variation (CV) for cathelicidin LL-37 measurements was high but after the introduction of two extra washes, the intra-assay CV was < 10%.

Statistical analysis

Statistical analyses were performed using SPSS version 20 (SPSS Inc., Chicago, IL, USA), GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, CA, USA), and R version 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria) [19]. Contingency tables were analysed using the χ^2 or Fisher's exact tests. Odds ratios with 99% confidence intervals (CIs) were calculated to measure the effect size.

Median values were compared using the Mann–Whitney test, and means were compared using the unpaired Student's *t*-test. Pairwise comparisons of non-parametric data were performed using the Kruskal–Wallis test. UHC analysis of biomarkers in CSF and serum, heat map generation, and PCA were performed using Qlucore Omics Explorer software (Qlucore AB, Lund, Sweden). Biological pathway analysis was performed using Ingenuity Pathway Analysis (Ingenuity Systems Inc., Redwood City, CA, USA). For the diagnostic prediction model, multivariable logistic regression analysis was used. Restricted cubic spline curve analysis was done to evaluate linearity of the factors. As none of the selected biomarkers showed linearity and sample size was limited, variables were dichotomized using the maximum value of the Youden-index (*J* = Sensitivity + Specificity – 1) as cut-off. Stepwise backward selection based on the Wald test (with $p = 0.10$ as the cut-off) was used to select variables. All possible combinations of the individual predictors were evaluated. The goodness of fit of the model was tested with the Hosmer–Lemeshow test. The receiver operating characteristic (ROC) curve based on the predicted probabilities of the model was calculated. Area under the curve (AUC), sensitivity, specificity, and positive and negative predictive values were calculated. Bootstrapping techniques were used for internal validation of the prediction models.

All measurements were conducted in duplicate and the calculated mean of the measurements was used in the analyses. Laboratory personnel were blinded to the clinical information associated with the samples. In all analyses, $p < 0.01$ was considered statistically significant unless otherwise stated.

Ethics

The study was approved by the Human Research Ethics Committee of Stellenbosch University (study nr. N09/10/265). Written informed consent was obtained from all patients or their caregivers.

RESULTS

Baseline characteristics

One hundred and forty-six participants with suspected meningitis were eligible for analysis. We analysed CSF and serum samples from 56 patients with and 55 without TBM (Figure 1). Of the children categorized as VM (n=25), 18 had viral pathogens detected by PCR in their CSF. Six out of ten BM children had positive CSF cultures. The No Meningitis Group consisted of a heterogeneous group of patients without meningitis (e.g. seizure, gastro-enteritis, respiratory tract infection, Ear Nose and Throat infection, Urinary tract infection).

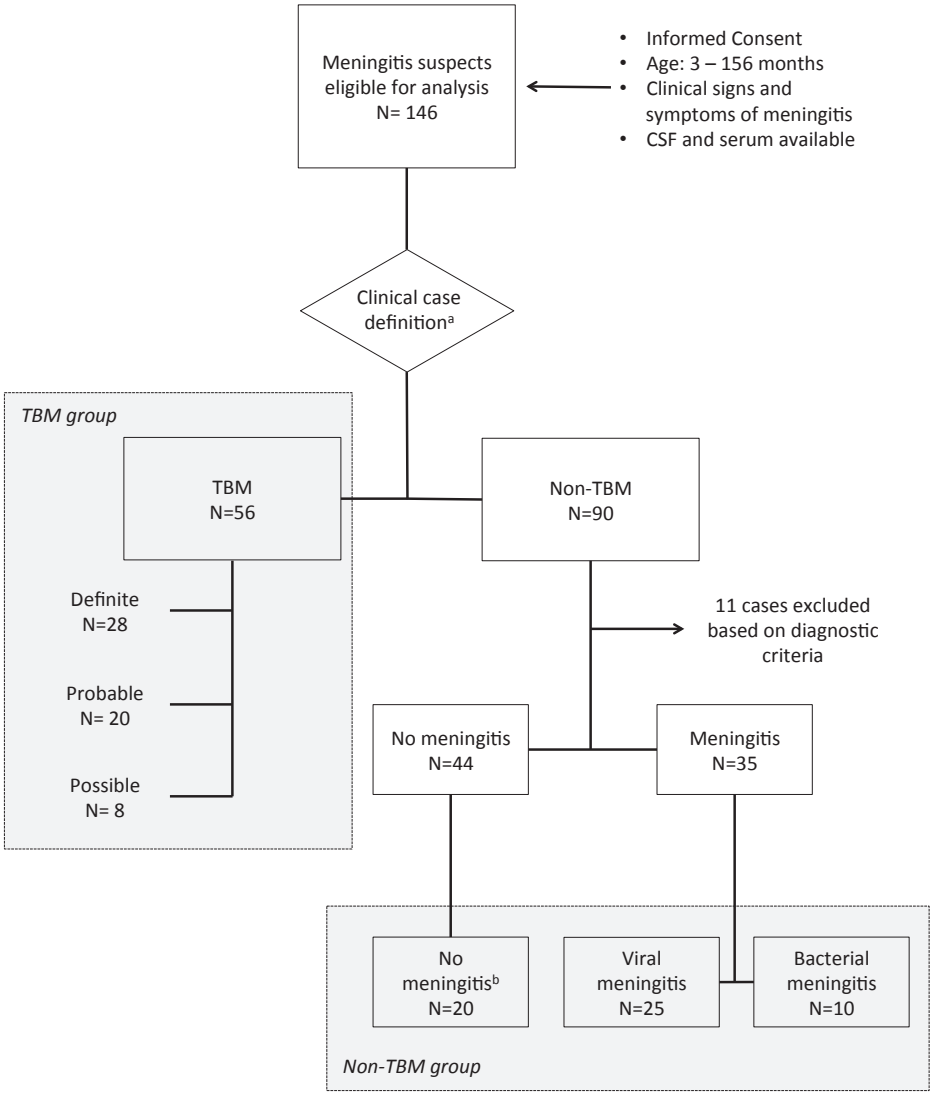


Figure 1: Flow diagram of the study participants

[a]Clinical case definition of Marais *et al* [15].

[b]Of the 44 patients without meningitis, samples of 20 patients were selected for analysis. Samples with the largest volume CSF and serum available were used.

Table 1: Baseline patient characteristics

Characteristic	TBM	Non-TBM		
	N (%)	N (%)	OR (99% CI)	p value
Total number of patients	56 (50.5)	55 (49.5)		
Age in months (mean ± SD)	49.1±39.9	56.9±43.7		0.33
Sex, male	30 (53.6)	35 (63.6)	0.66 (0.24–1.79)	0.28
HIV infected (n= 55; n=47)	4 (7.3)	3 (6.4)	0.87 (0.11–6.67)	0.86
BMI, < –2 SD (n=45; n=45) [a]	15 (33.3)	9 (20.0)	2.00 (0.57–7.04)	0.16
Race:				
Black	18 (32.1)	9 (16.4)	1.00	
Mixed ancestry	38 (67.9)	44 (80.0)	0.43 (0.13–1.43)	0.07
White [b]	0 (0)	2 (3.6)		
Presenting symptoms: [c]				
Fever	44 (78.6)	48 (87.3)	0.54 (0.14–2.04)	0.23
Headache	22 (39.3)	33 (60.0)	0.43 (0.16–1.17)	0.03
Convulsions	15 (26.8)	13 (23.6)	1.18 (0.38–3.65)	0.70
Vomiting	36 (64.3)	37 (67.3)	0.88 (0.31–2.46)	0.74
Focal neurological deficit	24 (42.9)	7 (12.7)	5.14 (1.47–18.0)	0.001
Irritability	22 (39.3)	18 (32.7)	1.33 (0.48–3.70)	0.47
Lethargy	21 (37.5)	16 (29.1)	1.46 (0.52–4.16)	0.35
Neck stiffness	33 (58.9)	28 (50.9)	1.38 (0.52–3.71)	0.40
Altered consciousness	39 (69.6)	23 (41.8)	3.19 (1.14–8.92)	0.004
Symptom duration >5 days	27 (48.2)	1 (1.8)	50.28 (3.42–740.12)	<0.001

n = 111 unless otherwise stated

[a]Standard deviation of body mass index values were based on the World Health Organization Child Growth Standards [20].

[b]Due to the limited number of white patients, these patients were excluded from analysis in this table.

[c]Presenting symptoms: more than one symptom seen in most cases.

Patient characteristics and presenting symptoms are outlined in Table 1. Altered consciousness, focal neurological deficits, and symptom duration > 5 days were statistically significant more present in the TBM Group. Table 1 comprises a detailed description of the TBM Group. Baseline concentrations of the 28 soluble mediators in serum and CSF of TBM and non-TBM cases are presented in Figure 2. Subgroup analysis identified significantly elevated concentrations of IL-13, VEGF, and cathelicidin LL-37 ($p < 0.05$), and a lower concentration of IL-17, in the CSF of patients with TBM compared to that of patients with VM and BM. In the serum of patients with TBM, concentrations of IL-17, cathelicidin LL-37, IFN-γ, and bFGF were significantly elevated ($p < 0.05$) compared with that of patients with VM and BM. These significant differences are detailed in Table 3.

Table 2: Characteristics of tuberculous meningitis patients

		N (%)
Total N		56 (100)
History		
Symptom duration >5 days		27 (48.2)
Symptoms suggestive of TB [a]		16 (28.6)
TB contact in history [b] (n=55)		29 (52.7)
Clinical signs		
TBM-stage [c] (n=55)	I	11 (20.0)
	IIa	16 (29.1)
	IIb	17 (30.9)
	III	11 (20.0)
Focal neurological deficit (excl. cranial nerve palsy)		18 (32.1)
Cranial nerve palsy		21 (37.5)
CSF		
Clear appearance		49 (87.5)
Leucocytes 10-500 cells/μL (n=55)		49 (89.1)
Lymphocytes >50%		52 (94.5)
Protein concentrations > 1 g/L (n=47)		33 (70.2)
Glucose <2.2 mmol/L (n=48)		29 (60.4)
Cerebral imaging		
Hydrocephalus (n=55)		43 (78.2)
Basal meningeal enhancement (n=55)		40 (72.7)
Tuberculoma (n=55)		13 (23.6)
Infarct (n=55)		18 (32.7)
Pre-contrast basal hyperdensity (n=52)		22 (42.3)

n = 56 unless otherwise stated; TBM – tuberculous meningitis; TB – tuberculosis; CSF – cerebrospinal fluid; TST – tuberculin skin test; IGRA –interferon gamma-release assay.

[a] Systemic symptoms suggestive of TB (1 or more of): weight loss/(poor weight gain in children), night sweats or persistent cough > 2 weeks

[b] History of recent close contact with an individual with pulmonary TB or a positive TST/IGRA in a child <10 years

[c] Refined TBM stage of van Toorn *et al* [21].

Subgroup analysis (Kruskal-Wallis Test) of the TBM Group did not show any significant difference in biomarker expression in CSF and serum between definitive, probable and possible TBM cases. Eight TBM patients received antimicrobial therapy (including adjunctive corticosteroids) prior to lumbar puncture. No significant differences were found in biomarker expression in CSF or serum between these patients and those that did not receive antimicrobial therapy prior to lumbar puncture.

Table 3: Differences in biomarker expression in cerebrospinal fluid and serum between patients with tuberculous, viral, and bacterial meningitis

	TBM	VM and BM		p value[a]
	Median [IQR] in pg/mL		Median [IQR] in pg/mL	
CSF				
IL-13	78.07 [50.50–104.82]	VM	26.24 [17.74–64.71]	0.001
		BM	29.15 [20.63–35.69]	0.003
IL-17	1.95 [0.00–6.05]	VM	38.73 [3.07–71.34]	<0.001
		BM	39.81 [2.18–93.88]	0.018
VEGF	142.81 [28.05–225.66]	VM	27.92 [7.92–48.73]	0.003
		BM	14.46 [8.70–86.47]	0.012
LL-37	5046.41 [3297.51–6194.68]	VM	2633.04 [479.73–4733.16]	0.015
		BM	142.46 [73.11–1297.42]	<0.001
Serum				
IL-7	23.79 [17.22–30.61]	VM	17.19 [12.37–21.73]	0.017
		BM	14.98 [8.25–22.77]	0.040
IL-6	32.30 [26.03–48.35]	VM	20.01 [13.60–28.74]	0.007
		BM	116.79 [38.36–206.04]	0.046
IL-17	123.55 [75.21–218.11]	VM	63.73 [37.65–79.90]	<0.001
		BM	40.07 [22.06–86.77]	0.004
LL-37 [b]	5052.18 [1961.05–10659.69]	VM [b]	1863.23 [1223.10–2287.02]	<0.001
		BM [b]	1082.34 [789.78–1873.04]	0.002
IFN-γ	478.33 [391.08–624.43]	VM	338.87 [157.26–416.32]	<0.001
		BM	222.34 [85.04–397.51]	0.005
bFGF	150.43 [94.95–222.17]	VM	94.84 [66.08–109.90]	0.001
		BM	74.64 [64.18–111.97]	0.009

[a]Kruskal–Wallis test with pairwise comparison; asymptotic significance two-sided tests with $p < 0.05$. Only statistically significant differences are presented.
[b]Cathelicidin LL-37 is presented in μg/mL instead of pg/mL.

Unsupervised hierarchical clustering and principal component analyses

To identify a TBM-specific biomarker profile, we performed UHC analysis and PCA. Significant clustering of patients with TBM was evident in the analysis of CSF samples (Figure 3a) but not serum samples. The results of UHC analysis of CSF samples were confirmed by PCA (Figure 3b). To determine the disease-specific biomarker signature of CSF, we calculated the grouped medians of each type of meningitis (TBM, VM, and BM) and the No Meningitis Group, and repeated UHC analysis. Highly significant distinct biomarker profiles were found to exist in the four subgroups (Figure 4). UHC analysis of the TBM Group did not show significant clustering of the three TBM subgroups (definitive, probable and possible TBM) in CSF and serum samples.

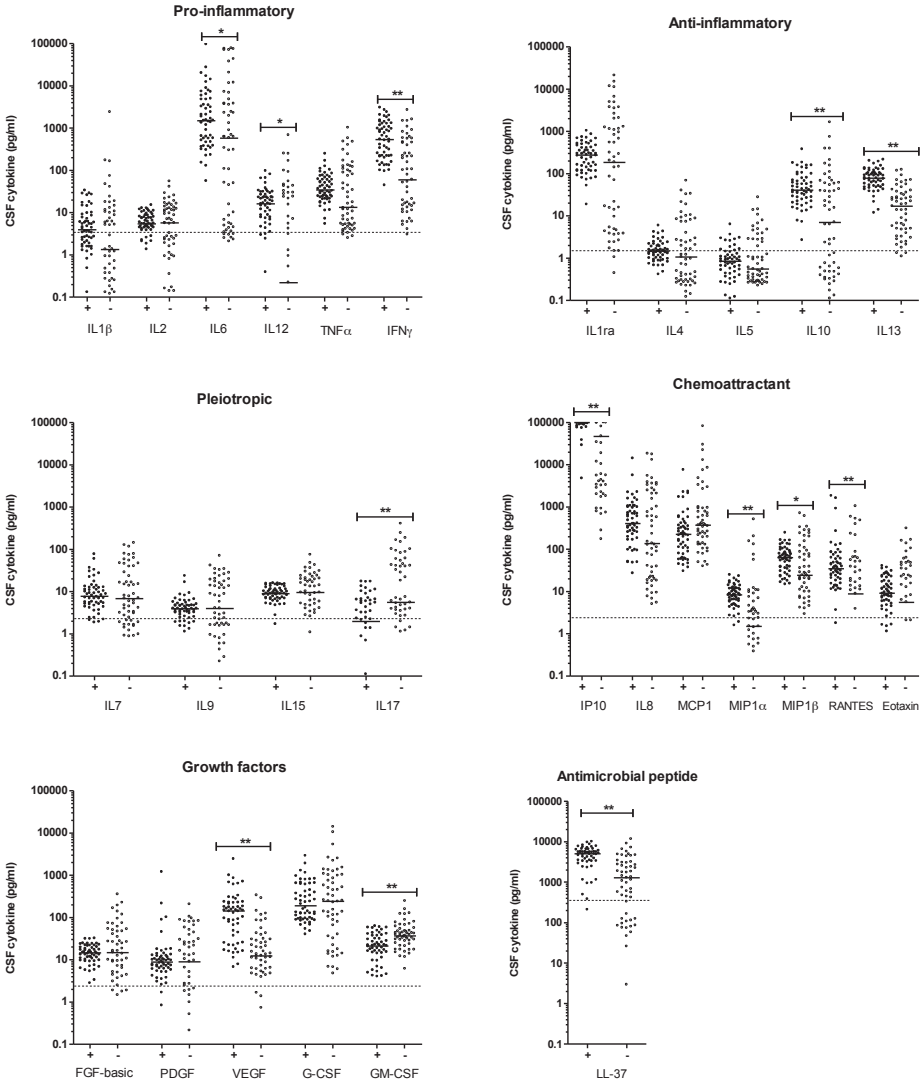
Ingenuity Pathway Analysis

To understand the biological relevance of the biomarkers differentially expressed in the CSF of patients with TBM, BM, and VM, we performed ingenuity pathway analysis. Pathways associated with IL-17 signalling, hypercytokinemia, and communication between immune cells were relevant to all three types of meningitis. However, the relative abundance of markers linked to pathways associated with pathogenesis of multiple sclerosis (MS), vitamin D receptor (VDR)/retinoid X receptor (RXR) activation, and CC chemokine receptor (CCR) 5 signalling in macrophages were greater in TBM than in BM or VM. Four markers involved in pathways associated with MS were upregulated in the CSF of patients with TBM (MIP-1α, MIP-1β, RANTES, and IP-10); however, in patients with BM, only RANTES, and in patients with VM, only MIP-1β, were upregulated. Similarly, the relative abundance of cytokines involved in the VDR pathway was greater in patients with TBM (cathelicidin LL-37, RANTES, IP-10, and IFN-γ) compared to patients with BM (RANTES and IL-2) and VM (G-CSF, IFN-γ, and IL-2). Three markers in patients with TBM (MIP-1α, MIP-1β, and RANTES) were associated with CCR5 signalling in macrophages, whereas only one of these molecules was present in relatively high abundance in patients with BM (RANTES) and VM (MIP-1β). This analysis suggests that distinct signalling pathways are activated in TBM, BM, and VM; however some discretion in drawing conclusions from this analysis is necessary as the BM group is relatively small in numbers (n=10).

Diagnostic model for tuberculous meningitis

A TBM-specific biomarker signature was found in CSF samples by UHC, PCA, and pathway analysis; therefore, we evaluated a biomarker-based diagnostic model for TBM. CSF biomarkers with significant differences in expression between TBM and the other meningitis subtypes (VM and BM) were used for this analysis (Table 3). The final diagnostic model consisted of a combination of three biomarkers: IL-13, VEGF, and cathelicidin LL-37. A ROC curve based on the predicted probability of the model was calculated, with an AUC of 0.93 (95% CI: 0.88–0.98). After internal validation, the AUC was 0.92 and regression coefficient was adjusted by a correction factor of 0.912. The final model had a sensitivity of 0.52 and a specificity of 0.95. The sensitivity, specificity, positive predictive value, and negative predictive value of the three biomarkers, both individually and in combination, are outlined in Table 4. The association of the three biomarkers used in the diagnostic model with clinical characteristics of TBM patients (Table 2) was evaluated with nonparametric testing. VEGF concentrations in CSF were significant higher in patients with CSF protein concentration > 1 g/L ($p = 0.009$), hydrocephalus ($p = 0.001$), basal meningeal enhancement ($p = 0.005$) and pre-contrast basal hyperdensity ($p = 0.009$). IL-13 levels were significantly higher ($p = 0.002$) in patients with CSF glucose < 2.2 mmol/L.

A


Pro-inflammatory
CSF cytokine (pg/ml)
IL1β
IL2
IL6
IL12
TNFα
IFNγ
Anti-inflammatory
IL1ra
IL4
IL5
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Pleiotropic
IL7
IL9
IL15
IL17
Chemoattractant
IP10
IL8
MCP1
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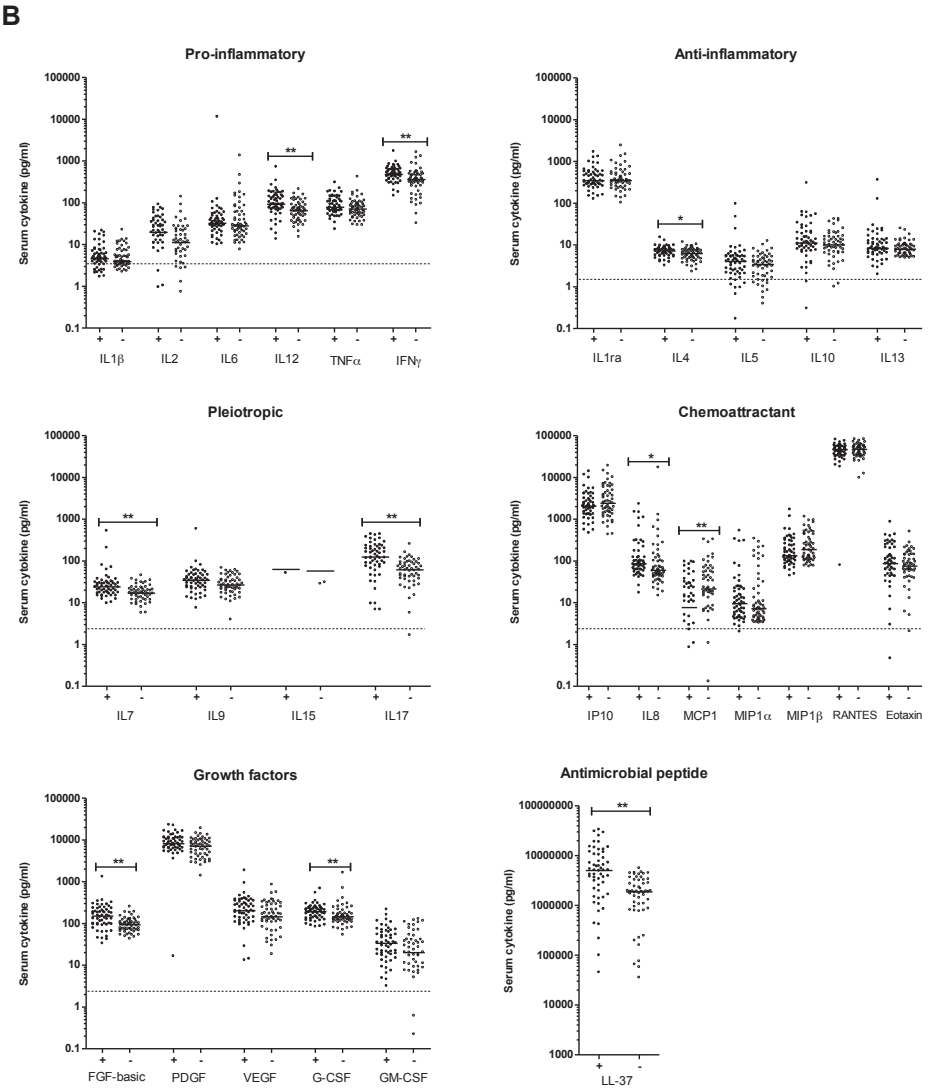

Figure 2: Cerebrospinal fluid and serum biomarker expression levels in patients with and without tuberculous meningitis

(**A**) CSF samples. (**B**) Serum samples. Results are expressed as pg/mL. Black circles (+) correspond to patients with tuberculous meningitis (TBM; *n* = 56); open circles (–) correspond to non-TBM patients (*n* = 55). Dotted lines represent the average limit of detection for the grouped cytokines. Statistical comparisons were made using the Mann–Whitney test (*p < 0.01 and ≥ 0.001; **p < 0.001).

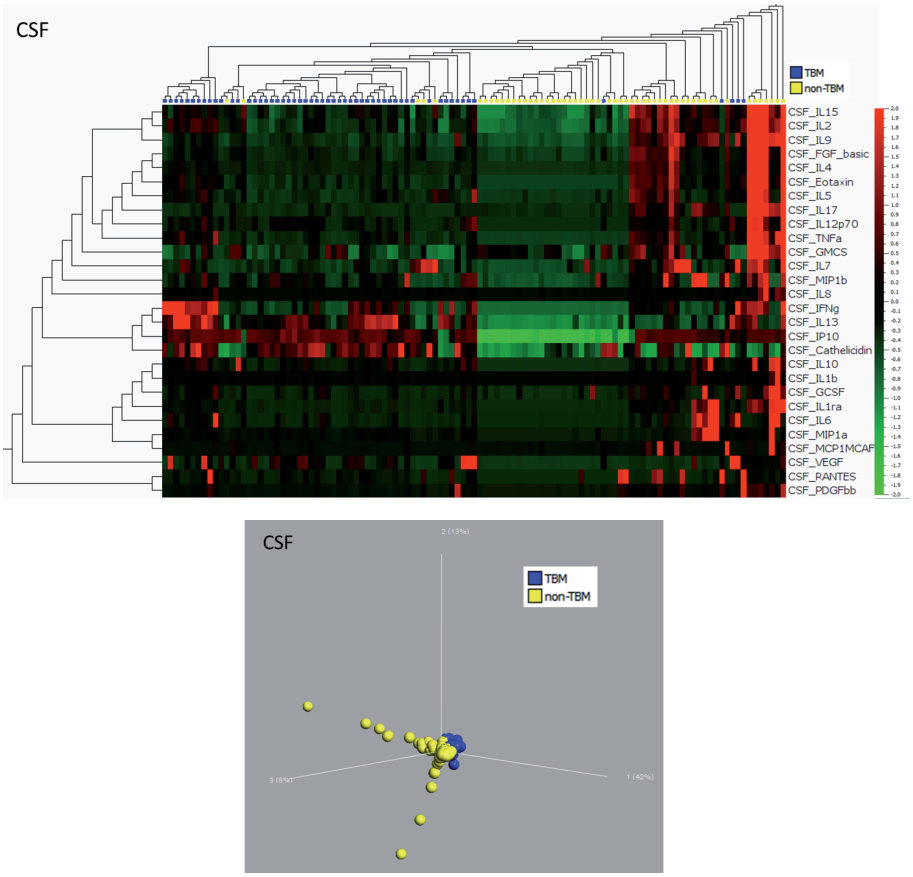

Figure 3: Unsupervised hierarchical clustering and principal component analyses of biomarkers in the cerebrospinal fluid of patients with and without tuberculous meningitis

(**A**) Two-dimensional unsupervised hierarchical clustering of biomarker profiles in the cerebrospinal fluid (CSF) of patients with (n = 56) and without (n = 55) tuberculous meningitis (TBM). The normalised values for each biomarker are depicted according to the colour scale, where red and green represent expression above and below the median, respectively. The dendrogram (above) shows the proximity between the different patients (blue = TBM; yellow = non-TBM), suggesting that patients within each subcluster probably share the same origin. Significant clustering of TBM cases is seen. (**B**) Three-dimensional representation of principal component analysis of patients with and without TBM. Each dot represents one participant based on the values of all biomarkers studied. The percentage of variance is depicted on three axes. The distance in space between each dot represents the relatedness between each individual. Visual clustering of TBM cases is seen.

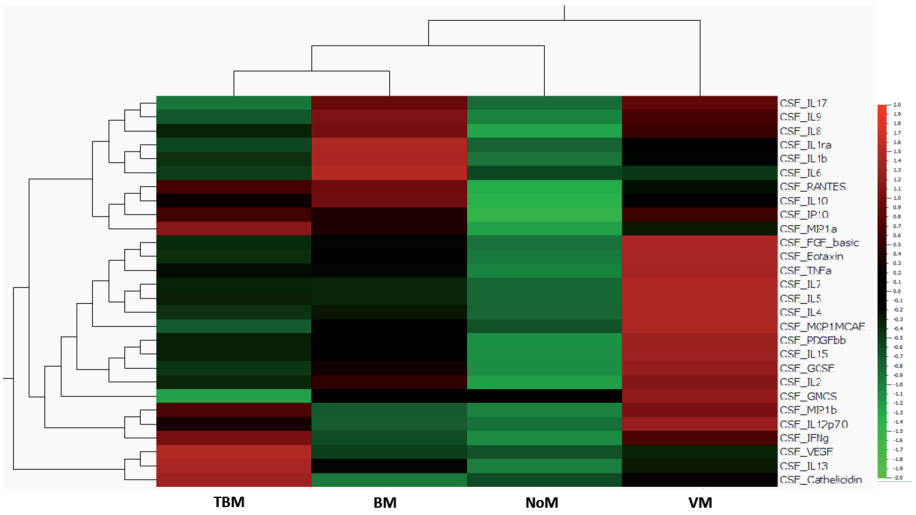

Figure 4: Biomarker expression profiles in the cerebrospinal fluid of patients with and in subgroups of patients without tuberculous meningitis

Unsupervised hierarchical clustering analysis of biomarker expression profiles in the cerebrospinal fluid of patients with (n = 56) and subgroups of patients without (Bacterial Meningitis Group [n = 10]; Viral Meningitis Group [n = 25]; and No Meningitis Group [n = 20]) tuberculous meningitis. Grouped medians per subgroup were taken to illustrate the differences in biomarker expression profiles between the groups.

Table 4: Cerebrospinal fluid biomarker-based diagnostic models for tuberculous meningitis

Positive biomarker	*B*	Sensitivity	Specificity	PPV	NPV
VEGF	1.46	1	0.60	0.72	1.00
LL-37	2.07	1	0.66	0.75	1.00
IL-13	2.63	0.96	0.78	0.82	0.95
VEGF and LL-37	3.53	0.93	0.86	0.87	0.92
VEGF and IL-13	4.09	0.89	0.86	0.87	0.88
IL-13 and LL-37	4.70	0.71	0.91	0.89	0.76
IL-13 and LL-37 and VEGF	6.16	0.52	0.95	0.91	0.66

Regression coefficients (B) were adjusted by a correction factor of 0.912 resulting in a corrected intercept of –3.679. Cut-off values were based on maximum Youden index (J = Sensitivity + Specificity – 1): vascular endothelial growth factor, 42.92 pg/mL; cathelicidin LL-37, 3221.01 pg/mL; interleukin-13 37.26 pg/mL. PPV: positive predictive value; NPV: negative predictive value.

DISCUSSION

We evaluated the biomarker expression profile of children with signs and symptoms suggestive of meningitis by investigating 28 biomarkers in their CSF and serum with multiple statistical methods. Using UHC and PCA, we identified a highly specific biomarker pattern in the CSF of patients with TBM compared with other types of meningitis. This biomarker pattern suggests a disease-specific host immune response, and could be of diagnostic and therapeutic value. Pathway analysis provided insight into the pathways upregulated in TBM. In the CSF of patients with TBM, the relative abundance of cytokines and chemokines involved in pathways related to the pathogenesis of MS (including CCR5 signalling in macrophages) and VDR/RCR activation was greater than in the CSF of patients with BM or VM. MS is a chronic demyelinating disease of the CNS with several diverse pathogenic mechanisms. Influx of activated T cells and macrophages into the CNS is considered an important step in MS development. Release of pro-inflammatory cytokines by T helper (Th) 1 cells and macrophages triggers a chain of events, resulting in the formation of demyelinated plaques and axonal damage [22]. A network of chemokines and chemokine receptors, including MIP-1α, MIP-1β, RANTES, IP-10, and CCR5, may influence the trafficking of immune cells and their transfer to lesion sites, and is a potential target for the treatment of MS [22]. The upregulation of MS-related signalling in TBM suggests that similar treatment modalities could affect the sequelae of TBM.

The active form of vitamin D, 1,25-dihydroxyvitamin D_3 (1,25D), has physiological functions that extend beyond its classical role in calcium homeostasis and bone metabolism. Vitamin D deficiency is associated with inflammatory diseases and disorders with long latency periods, such as MS, rheumatoid arthritis, diabetes, and tuberculosis [23, 24]. The involvement of components of the VDR/RXR pathway supports a putative role for vitamin D in the pathogenesis of TBM [25] and is reminiscent of autoimmune diseases, such as MS.

The current reference standard for diagnosing TBM is the visualisation of acid-fast bacilli in and/or culture of *M. tuberculosis* from the CSF. Mycobacterial culture of CSF may take up to 42 days to receive the results and both having low sensitivities (10-20%) [26, 27]. Many attempts have been made to develop simplified, mostly antigen-detection tests for TB but their diagnostic power remains poor [28]. Recently, a multidisciplinary group of TB experts proposed the essential criteria for a point-of-care test [29]. For extrapulmonary TB, the test should provide a sensitivity of 60% for probable TBM and a specificity of 95%. From this perspective, the diagnostic model presented here (sensitivity 52%, specificity 95%), although approaching that performance, falls short as a point-of-care test for the diagnosis of TBM. However, when compared to the current standard of diagnostic tests for childhood TBM,

which have an even lower sensitivity, further refinement of this model appears important. Misra UK et al. found infarctions to be associated with relative increased VEGF concentrations in CSF of TBM patients [30]. Interestingly we did not find this association but found clear associations with CSF protein concentration > 1 g/L, hydrocephalus, basal meningeal enhancement and pre-contrast basal hyperdensity. VEGF is a potent inducer of vascular permeability and angiogenesis [31] and is implicated in the pathogenesis of cerebral oedema related to ischaemia, trauma, tumours, and infection [32, 33]. Several studies report elevated levels of VEGF in the CSF, [34-36] suggesting that it may contribute to disruption of the blood–brain barrier and cerebral oedema formation in TBM.

Our study has certain limitations. First, only 28 host markers were evaluated in this study, and numerous additional components are evaluable with multiplex cytokine arrays and should be included in future studies. Although internal validation in the same patient cohort is necessary and helpful, the ability to generalise the prediction model in an independent group of patients is necessary. Therefore, external validation with new data from an independent, comparable population at a different location should be performed prior to the implementation of this model in the clinical setting. Second, a case definition based on consensus of expert opinion was used for TBM diagnosis and could have caused misclassification bias. Interestingly subgroup analysis of the 56 TBM patients revealed no immunological difference between definitive, probable and possible TBM cases. Third, eight TBM patients received antimicrobial therapy (including adjunctive corticosteroids) prior to lumbar puncture. Since several years, corticosteroids have been used as adjunct to antituberculous drugs to improve outcome in HIV-negative people [37] but the exact mechanisms are still incompletely understood. Simmons CP et al. [38] studied the kinetics of the inflammatory response in cerebrospinal fluid and peripheral blood from 87 adults with TBM and found only significant modulation of acute CSF protein concentrations and marginally reduced IFN-γ concentrations. Other immunological and routine biochemical indices of inflammation were unaffected, similar to the results presented in the study herein.

In conclusion, this study improves our understanding of the pathogenesis of TBM and may direct future strategies for the prevention of immunopathology associated with this devastating disease. Our data suggest that host biomarker signatures in the CSF have promising diagnostic applications and require urgent investigation.

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3 Seasonal variation in the incidence rate of tuberculous meningitis is associated with sunshine hours

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ABSTRACT

Tuberculous meningitis (TBM) is a severe complication of tuberculosis and occurs mainly during early childhood. The incidence rate of TBM varies with season, and serum vitamin D levels, which are dependent on sunlight, might play a role. We studied the association between TBM incidence rate and hours of sunshine in Cape Town, South Africa and found a significant association between the incidence rate of TBM and sunshine hours 3 months earlier (Incidence rate ratio (IRR) per 100 sunshine hours, 0.69; 95%CI 0.54–0.88; P 0.002). The association supports the hypothesis that vitamin D might play a role in the pathophysiology of TBM. Further prospective studies in which vitamin D status is measured are necessary to determine causality.

INTRODUCTION

One-third of the world's population is currently infected with *Mycobacterium* (*M.*) *tuberculosis*, and each year more than 1.5 million people die as a result of this disease [1]. Tuberculous meningitis (TBM) is the most severe extrapulmonary complication of tuberculosis and occurs mainly during early childhood. Haematogenous spread of bacilli from a primary pulmonary focus can lead to the development of a caseous granuloma in the meninges or brain adjacent to the meninges or ventricular ependymal. Rupture of this so-called Rich focus into the subarachnoid space causes the clinical features of TBM. In the majority of cases, TBM develops within a few months after the primary infection [2].

Seasonal variation in the incidence of disease caused by *M. tuberculosis* (including TBM) has been described in the literature, but the exact cause of this phenomenon remains unknown [3, 4]. Intensified transmission during winter periods, under conditions of overcrowded, poorly ventilated housing, might play a role [3]. Vitamin D deficiency, which is known to be associated with progression of *M. tuberculosis* disease [5], has also been suggested to play a role [4]. However, a possible association between vitamin D deficiency and TBM has never been evaluated. If vitamin D deficiency does play a role in the pathophysiology of TBM, one would expect an association between sunshine hours during the period before manifestation of the disease and the incidence rate of TBM.

METHODS

Consecutive children between 6 months and 13 years old, diagnosed with 'definite' or 'probable' TBM at a large tertiary teaching hospital in Cape Town, South Africa between April 2000 and April 2005, were included retrospectively in the present study. Clinical data on these children were derived from a TBM-study database published by van Well et al. [6]. A 'definite' diagnosis of TBM was made when *M. tuberculosis* was isolated from the cerebrospinal fluid (CSF). A 'probable' diagnosis of TBM was made when there were clinical signs of meningitis in the presence of characteristic CSF findings (macroscopically clear, pleiocytosis, elevated protein, reduced glucose) and two or more of the following criteria had to be present:

1. Recent poor weight gain
2. Household contact with sputum smear-positive TB
3. Computed Tomography scan compatible with TBM
4. Chest radiography compatible with primary TB
5. Positive tuberculin skin test (TST)
6. Acid-fast bacilli cultured from other clinical specimens [6].

The dates of admission were used to estimate the monthly incidence rate of TBM. Monthly sunshine hours between April 2000 and April 2005 were obtained from the South African Weather Service and used for analysis. Sunshine hours were measured with a Campbell–Stokes sunshine recorder at Cape Town International Airport (coordinates: 33.97° South, 18.60° East; 42 m above sea level). Sunshine hours were defined as the total sum of hours during a certain period of time when the direct solar radiance exceeded 70 W/m^2 in very dry air and 280 W/m^2 in very humid air. Ultraviolet-B (UVB) radiation (280–315 nm) was measured with a Solar® Light UV-Biometer, model 501 at the Global Atmosphere Watch (GAW) station, Cape Point (coordinates: 34.35° South, 18.49° East; 230 m above sea level) between February 2001 and April 2005. The UV-Biometer used is a meteorological grade instrument that measures biologically effective UVB radiation outdoors. Monthly UVB radiation was expressed in terms of the minimum erythema dose (MED); one MED is defined as 583 W/m^2 falling continuously for 1 hour.

To study the impact of monthly sunshine hours and UVB radiation on the incidence rate of TBM, the log–linear Poisson regression model was used. Incidence rate ratios (IRRs) with accompanying 95% confidence intervals (95%CI) were used as measures of relative risk. Time intervals of 1 month, counted from the month of admission backwards to 6 months before admission, were used for the analysis. Statistical significance was determined at the 1% level. Patient characteristics (sex, age, population group, HIV status, and BCG scar) were considered as potential confounding factors. The patients were divided into three population groups: white (European descent), black (African descent), and coloured (people of mixed descent, including Asiatic descent). Confounders that led to a change in the coefficient of more than 10% were added to the model. The Statistical Package for the Social Sciences version 18.0 for Macintosh (IBM®) was used for the statistical analysis.

RESULTS

In total, 189 children aged between 6 months and 13 years were diagnosed with TBM between April 2000 and April 2005. The characteristics of the patients are summarized in Table 1. The highest number of sunshine hours was measured during the summer season (December to February), with a maximum of 361.90 hours/month. The lowest number of sunshine hours was measured during the winter months (June to August), with a minimum of 165.50 hours/month. The level of UVB radiation was comparable to the hours of sunshine during the summer season (maximum 335.86 MED/month), but during the winter months the level of UVB showed a steeper decline than that of

sunshine hours (minimum 20.29 MED/month). A significant association was found between the monthly incidence rate of TBM and the number of hours of sunshine 3 months earlier (IRR per 100 sunshine hours, 0.69; 95%CI 0.54–0.88; P 0.002). This implies that a decrease of 100 sunshine hours/month was associated with a 45.0% (= 1/0.69) increase in TBM incidence 3 months later (Figure 1). Similar results were found for UVB radiation (IRR per 100 MED, 0.75; 95%CI 0.61–0.91; P 0.003).

Table 1 Characteristics of children admitted with a diagnosis of TBM between April 2000 and April 2005.

	Total
Characteristics	n=189 (100%)
Age (median)*	28.0 (15.0–47.5)
Sex (male)	69 (50.8)
Population group	
Black	42 (22.2)
Coloured	147 (77.8)
White	0 (0)
TBM stage**	
I	0 (0)
II	91(48.1)
III	98 (51.9)
BCG scar (n=187)	27 (14.4)
HIV positive (n=135)	7 (5.2)

* Age in months, (Interquartile range)

** TBM was staged using the modified criteria of the British Medical Research Council to determine the severity of TBM: stage I TBM [Glasgow coma score (GCS) 15 with no focal neurological signs], stage II TBM (GCS 11–14 or GCS of 15 with focal neurological deficit) and stage III TBM (GCS < 11).

DISCUSSION

This is the first study to demonstrate an association between the incidence rate of TBM and sunshine hours. Low amounts of sunshine during the winter months were associated with an increase in TBM incidence 3 months later. This finding suggests a possible role for vitamin D in the pathophysiology of TBM.

In the presence of UVB radiation, vitamin D3 is synthesized from a precursor in the skin (7-dehydrocholesterol). In general, solar UVB radiation is expressed in terms of the MED, which is defined as the dose of UVB irradiation which, after falling continuously, will start to damage human skin. The MED is a personal value and is dependent on age and skin type. During the winter months in Cape Town, the intensity of

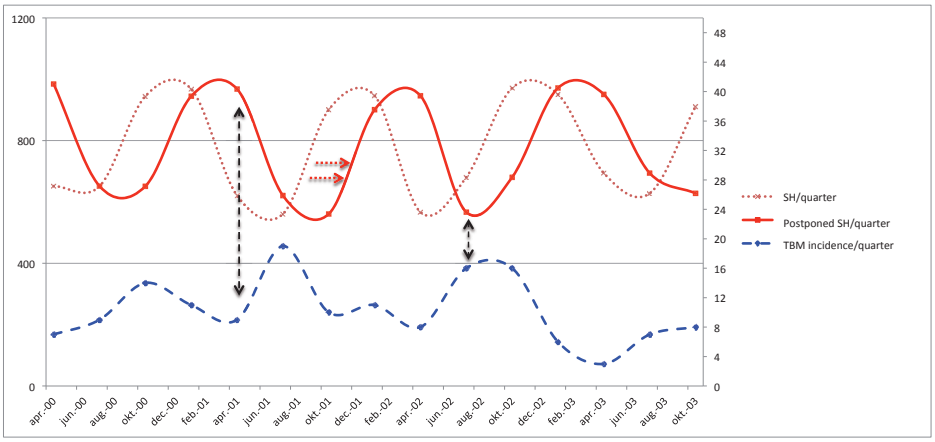

Figure 1:
Incidence rate of TBM (blue dotted curve; right y-axis) and sunshine hours (SH; red dashed curve; left y-axis) are shown per quarter (= 3 months). The solid red curve (postponed sunshine hours) illustrates the inverse association with the incidence rate of TBM. Low amounts of sunshine during winter months lead to an increase in TBM incidence (black arrows). For reasons of clarity only part of the graph is shown.

UVB radiation declines to approximately 1 MED a day, which can lead to insufficient vitamin D production, especially in persons with a dark pigmented skin [7-9].

The active form of vitamin D, 1.25-dihydroxyvitamin D3 (1.25D), has physiological functions that extend beyond its classical role in calcium homeostasis and bone metabolism. Over the last two decades, vitamin D deficiency has been found to be associated strongly with inflammatory diseases and those of long latency, such as multiple sclerosis, rheumatoid arthritis, diabetes, and *M. tuberculosis* disease [5, 10]. Once converted, 1.25D binds to the intracellular vitamin D receptor (VDR). This nuclear receptor is found in several cells that are involved in the human immune system, including stimulated macrophages, T-cells, and B-cells. Activation of the VDR influences patterns of cytokine secretion, suppresses T-cell activation, and can enhance the phagocytic activity of macrophages [11].

Activation of monocytes by *M. tuberculosis* through the Toll-like receptor can lead to VDR-dependent production of pro- and anti-inflammatory cytokines and the expression of antimicrobial peptides, such as cathelicidin, that have direct antimicrobial activity [11]. Recently, it was demonstrated that the production of proinflammatory cytokines, such as tumour necrosis factor alpha, interleukin (IL)-6, IL-1β, and interferon gamma, shows seasonal variation and is dependent on vitamin D [12]. Martineau et al. showed that there is a reciprocal seasonal relationship between serum vitamin D concentration and TB notifications in Cape Town, South Africa and

concluded that seasonal variations in vitamin D status and TB incidence are directly causally related [9].

Until now no studies have been published on vitamin D and its role in the pathophysiology of TBM. However, it is known that microglial cells, which play a key role in TBM, express VDR on the surface of the nucleus [13]. In rats, in vitro stimulation of these microglial cells with lipopolysaccharide results in the synthesis of 1,25D [14].

We accept that there are certain limitations to our study. Given that it was a retrospective study we could not obtain information on vitamin D status or factors that could possibly have influenced serum vitamin D levels (i.e. diet, body mass index, co-morbidity, socio-economic status, and personal exposure to sunlight). Intensified transmission of disease during winter periods owing to overcrowded and poorly ventilated housing conditions, as mentioned before, cannot be ruled out as a confounder in this association. Therefore, the involvement of vitamin D in the association of the incidence rate of TBM with sunshine hours is only speculative.

However, an association between sunshine hours and TBM incidence rate has been described for the first time in the present study. This association supports the hypothesis that vitamin D might play a role in the pathophysiology of TBM. Further prospective studies in which vitamin D status is measured are necessary to determine causality.

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4 Exploring the role of vitamin D and cathelicidin LL-37 in the pathophysiology of childhood tuberculous meningitis

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Submitted

ABSTRACT

Background – Growing evidence suggests that vitamin D is involved in the pathophysiology of tuberculosis (TB); low serum 25-hydroxyvitamin D (25(OH)D) levels are associated with increased active TB risk. The antimicrobial peptide cathelicidin LL-37 is important in the host immune response to *Mycobacterium tuberculosis* and its production is dependent on 25(OH)D. To date, no studies have explored the role of vitamin D or cathelicidin LL-37 in the pathophysiology of tuberculous meningitis (TBM).

Methods – We conducted a prospective, hospital-based study among children aged 3 months to 13 years with suspected meningitis and investigated serum 25(OH)D levels and cerebrospinal fluid (CSF) concentrations of cathelicidin LL-37 and five other vitamin D-related biomarkers.

Results – Serum and CSF samples from 97 patients with suspected meningitis were analysed of whom 48 patients were diagnosed with TBM. One third of the study population had serum 25(OH)D levels < 50 nmol/L. Serum 25(OH)D <75 nmol/L was presented more often in TBM than non-TBM patients (odds ratio 2.87, 95% confidence interval 1.14–7.22, $p = 0.03$). CSF cathelicidin LL-37 concentrations were high in TBM patients but did not correlate with serum 25(OH)D levels in contrast to non-TBM patients. Significant correlations existed between cathelicidin LL-37 and Interleukin-13, Interferon-γ (IFN-γ), Regulated on activation normal T cell expressed and secreted (RANTES) and IFN-γ-induced protein-10 in CSF.

Conclusions – Low serum 25(OH)D levels are associated with TBM in a paediatric population with suspected meningitis. The correlation between the CSF concentrations of vitamin D-related biomarkers and cathelicidin LL-37 stresses the role of vitamin D in the pathophysiology of TBM.

INTRODUCTION

Central nervous system involvement occurs in approximately 1% of all cases of tuberculosis (TB) [1]. Tuberculous meningitis (TBM) is the most severe form and frequently occurs during early childhood [2]. Haematogenous spread of bacilli from a primary pulmonary focus can induce the development of a caseous granuloma, or Rich focus, in the meninges, adjacent brain tissue, or ventricular ependyma. Rupture of a Rich focus into the subarachnoid space causes the clinical features of TBM. In most cases, TBM develops within a few months of the primary infection [3].

Hypovitaminosis D is widespread in developing countries [4] and is, alongside infectious diseases and malnutrition, among the most prevalent childhood health disorders [5]. Over the last two decades, low vitamin D levels have been found to be strongly associated with inflammatory diseases, particularly those of long latency, such as multiple sclerosis, rheumatoid arthritis, diabetes and *Mycobacterium (M.) tuberculosis* disease [6–8]. Since Rook *et al*. [9] demonstrated that the active form of vitamin D, 1,25-dihydroxyvitamin D3 (1,25D) inhibits *M. tuberculosis* replication, growing evidence suggests that vitamin D contributes to the pathophysiology of TB [10]: low serum vitamin D levels are associated with increased risk of active TB [8].

To date, no studies have explored the role of vitamin D in the pathophysiology of TBM. Microglial cells, which play a key role in TBM, were shown to synthesize 1,25D after *in vitro* stimulation with lipopolysaccharide (LPS) [11]. Once converted, 1,25D binds to the intracellular vitamin D receptor (VDR). This nuclear receptor is found in several cells involved in the human immune system, including microglial cells [12]. Activation of monocytes by *M. tuberculosis* through the Toll-like receptor (TLR) signalling pathway can cause VDR-dependent production of pro- and anti-inflammatory cytokines and expression of antimicrobial peptides, such as cathelicidin LL-37 [13]. Interleukin-13 (IL-13) and IL-15 are cytokines related to the vitamin D-mediated production of cathelicidin LL-37 [14, 15]. Recently, we demonstrated upregulation of cathelicidin LL-37 and biomarkers associated with VDR activation (Interferon [IFN]-γ, Regulated on activation normal T cell expressed and secreted [RANTES] and IFN-γ-induced protein-10 [IP-10]) in patients with TBM relative to patients with other types of meningitis [Visser DH *et al*., manuscript in preparation].

Cathelicidin LL-37 represents one of the antimicrobial components of macrophages and is a product of the Human cationic antimicrobial protein (*hCAP-18*). In TB, cathelicidin LL-37 induction in macrophages promotes the destruction of intracellular *M. tuberculosis*, [10] and directs macrophage differentiation towards a pro-inflammatory phenotype [16]. The positive correlation between serum 25-hydroxyvitamin D (25(OH)D) and cathelicidin LL-37 levels has been described in critically-ill adults admitted to the intensive care unit, [17] but this systemic correla-

tion was absent in serum of pulmonary TB cases [18, 19]. Whether there is a correlation between serum 25(OH)D and the localized production of cathelicidin LL-37 in cerebrospinal fluid (CSF) of TBM cases is unknown.

In this study, we investigated serum 25(OH)D levels in a paediatric cohort with suspected meningitis resident in an area where TB is endemic. We hypothesized that serum 25(OH)D levels are lower in patients with TBM compared with patients with other types of meningitis. Additionally, we investigated the relation between serum 25(OH)D level and the concentration of cathelicidin LL-37 in CSF. We anticipated that low serum 25(OH)D is associated with low cathelicidin LL-37 concentration in the CSF. Finally, we evaluated the correlation between CSF concentrations of cathelicidin LL-37 and five other vitamin D-related biomarkers.

METHODS

Study population and case definition

We conducted a prospective, hospital-based study among children aged 3 months to 13 years old with symptoms and signs suggestive of meningitis admitted to the Tygerberg Hospital, Cape Town, South Africa between November 2009 and November 2012. Blood and CSF samples were collected from all patients during a routine diagnostic workup. Samples were centrifuged, then CSF supernatant and serum were aliquoted into sterile polypropylene microtubes and stored at –80°C until analysis. Only patients with volumes of serum and CSF sufficient for analysis were enrolled.

Children with suspected meningitis were categorized retrospectively as TBM and non-TBM patients. Categorization was based on the uniform clinical case definition of Marais *et al*. [20]. In brief, TBM was classified as 'Definite' when acid-fast bacilli were evident in the CSF, *M. tuberculosis* was cultured from the CSF, or *M. tuberculosis* was detected by a nucleic acid amplification test in the CSF of a patient with symptoms or signs suggestive of the disease. TBM was classified as 'Probable' or 'Possible' on the basis of a scoring system comprising clinical, laboratory and radiological criteria [20]. Patients were classified as suffering from non-TBM disease when an alternative diagnosis was established and comprised patients with viral meningitis (VM), bacterial meningitis (BM) and a heterogeneous group of no meningitis patients. The VM Group comprised children with polymerase chain reaction-confirmed VM and those with a CSF leukocyte count $\geq 10^7$/L in the absence of microorganisms on Gram staining or routine culture and a clinical course consistent with VM [21]. The BM Group comprised patients with a positive CSF culture, evidence of bacteria on Gram staining, or a positive bacterial antigen latex agglutination test. It also included patients with features characteristic of BM in the CSF (high protein concentration, low glucose

concentration and polymorph predominance), with or without a positive blood culture for a bacterial pathogen and with a clinical course consistent with BM. The No Meningitis Group comprised patients with clinical signs and symptoms of meningitis but in whom meningitis was excluded on the basis of normal CSF and another diagnosis was apparent, with a clinical course consistent with this diagnosis at discharge.

Serum 25-hydroxyvitamin D levels

Serum 25(OH)D levels were measured using high-performance liquid chromatography followed by ultraviolet detection, in accordance with the manufacturer's protocol (RECIPE Chemicals + Instruments GmbH, Munich, Germany). The average inter-assay coefficient of variation (CV) was 8.7%. The cut-off used to define adequate serum vitamin D levels has been controversial for decades; however, in recent years, levels of 25(OH)D < 50 nmol/L have been considered to represent deficiency and 50 – 75 nmol/L as insufficiency [22–24]. In this study three different serum 25(OH)D cut-off values were used for analysis (25, 50 and 75 nmol/L).

Cathelicidin LL-37 and cytokine concentrations in CSF

Frozen samples were defrosted rapidly before analysis. CSF supernatant was passed through a hydrophilic Durapore® polyvinylidene difluoride filter membrane (MultiScreen$_{HTS}$ GV Plate 0.22 µm; EMD Millipore Corporation, Billerica, MA, USA). Cathelicidin LL-37 concentration was assessed using an enzyme-linked immunosorbent assay kit (USCN Life Science Inc., Houston, TX, USA) based on the manufacturer's protocol, with two extra wash steps to optimize the intra-assay CV.

Cytokine levels were measured using multiplex bead array technology according to the manufacturer's instructions (Bulletin#10014905; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Assays were read on the Bio-Plex 200 platform (Bio-Rad) and the Bio-Plex Manager software 6.0 was used for bead acquisition and analysis. The following biomarkers were analysed: IL-13, IL-15, IFN-γ, RANTES and IP-10. Measurements were conducted in duplicate and the calculated means of the measurements were used in the analyses.

Statistical analyses

Statistical analyses were performed using SPSS version 20.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA, USA). First, differences in clinical characteristics between children with TBM and children with non-TBM diseases were tested. Differences in frequencies were tested using the chi-squared or Fisher's exact tests. Odds ratios (OR) with 95% confidence intervals (CI) were calculated. Differences in non-parametric variables were tested using the Mann–Whitney *U* test and the data were expressed as medians and interquartile ranges. Second, differences in serum 25(OH)D levels between the TBM and

Table 1: Clinical characteristics of tuberculous meningitis and non-tuberculous meningitis patients

Characteristic	TBM (n=48)	Non-TBM (n=49)		
	N (%)	N (%)	OR (95% CI)	p value*
Total number of patients	48 (49.5)	49 (50.5)		
Age in months (median, IQR)	37 [22–66]	56 [24–94]		0.29
Sex, female	23 (47.9)	18 (36.7)	1.58 (0.70 – 3.57)	0.31
HIV-infected (n=47; n=41)	4 (8.5)	3 (7.3)	1.18 (0.25 – 5.60)	1.00
Multivitamins in last 6 months (n=47; n=49)	14 (29.8)	14 (28.6)	1.06 (0.44 – 2.56)	1.00
Formula milk in last 6 months (n=47; n=49)	34 (72.3)	39 (79.6)	0.67 (0.26 – 1.72)	0.48
BMI, < –2 SD (n=39; n=41) [a]	13 (33.3)	9 (22.0)	1.78 (0.66 – 4.81)	0.32
Formal housing	30 (62.5)	34 (69.4)	0.74 (0.32 – 1.71)	0.53
Substance use in the house:				
Alcohol	18 (37.5)	20 (40.8)	0.87 (0.39 – 1.97)	0.84
Smoking	25 (52.1)	32 (65.3)	0.58 (0.26 – 1.31)	0.22
Drugs	5 (10.4)	3 (6.1)	1.78 (0.40 – 7.92)	0.49
Monthly income (Rand) (median and IQR) (n=41; n=46)	1400.0 [490.0–2150.0]	1675.0 [487.5–3105.0]		0.28[d]
Child support grant	14 (29.2)	15 (30.6)	0.93 (0.39 – 2.23)	1.00
Caregiver's education (n=44; n=48)				0.21[e]
Grade ≤ 7	6 (13.6)	6 (12.5)		
Grade 8–9	21 (47.7)	15 (31.2)		
Grade ≥10	17 (38.6)	27 (56.2)		
Ethnicity				0.04[e]
Black African	16 (33.3)	7 (14.3)		
Mixed ancestry	32 (66.7)	40 (81.6)		
Other [b]	0 (0.0)	2 (4.1)		

Table 1: Clinical characteristics of tuberculous meningitis and non-tuberculous meningitis patients (continued)

Characteristic	TBM (n=48)	Non-TBM (n=49)		
	N (%)	N (%)	OR (95% CI)	p value*
Presenting symptoms: [c]				
Altered consciousness [f]	35 (72.9)	20 (40.8)	3.90 (1.66 – 9.17)	<0.01
Headache	20 (41.7)	32 (65.3)	0.38 (0.17 – 0.86)	0.03
Convulsions	10 (20.8)	12 (24.5)	0.81 (0.31 – 2.11)	0.81
Vomiting	29 (60.4)	34 (69.4)	0.67 (0.29 – 1.56)	0.40
Focal neurological deficits	19 (39.6)	7 (14.3)	3.93 (1.47 – 10.55)	<0.01
History of fever	37 (77.1)	42 (85.7)	0.56 (0.20 – 1.60)	0.31
Irritability	18 (37.5)	15 (30.6)	1.36 (0.59 – 3.16)	0.53
Lethargy	17 (35.4)	15 (30.6)	1.24 (0.53 – 2.90)	0.67
Neck stiffness	28 (58.3)	25 (51.0)	1.34 (0.60 – 3.00)	0.54
Symptom duration > 5 days	23 (47.9)	1 (2.0)	44.16 (5.63 – 346.36)	<0.01
Season of inclusion, October–March	24 (50.0)	37 (75.5)	0.32 (0.14 – 0.77)	0.01

n = 97 unless otherwise stated; * two-sided Fisher's exact test used unless otherwise stated.
[a] Standard deviation of body mass index (BMI) values was based on the World Health Organization Child Growth Standards [25].
[b] One Caucasian and one Asian patient; [c] Presenting symptoms: more than one symptom seen in most cases; [d] Mann–Whitney *U* test used; [e] Pearson's chi-squared test used; [f] Altered consciousness was defined as a Glasgow Coma Scale score < 15.

Non-TBM Groups were analysed using the independent samples *t*-test. Third, the association between serum 25(OH)D levels and TBM was evaluated using logistic regression analysis with the results adjusted for confounders that caused a change in the coefficient of > 10%. Finally, Spearman's rank correlation coefficient was used for correlation analyses between serum 25(OH)D levels and concentrations of cathelicidin LL-37 and between cathelicidin LL-37 and other CSF biomarkers. In all analyses, $p < 0.05$ (2-sided) was considered statistically significant.

Ethics

This study was conducted according to the ethical guidelines and principles of the *Declaration of Helsinki* and the South African guidelines for good clinical practice. Ethical approval was obtained from the Stellenbosch University Research Ethics Committee. The Department of Paediatrics and Child Health, Tygerberg Children's Hospital granted approval for the recruitment of study participants. Written informed consent was obtained from all patients or their caregivers.

RESULTS

Baseline characteristics of the study population

Serum and CSF samples from 97 patients with suspected meningitis were analysed: 48 patients were categorized as TBM and 49 as non-TBM diseases. The Non-TBM Group comprised 25 patients with VM, eight patients with BM and a heterogeneous group of 16 patients with suggestive signs and symptoms but in whom meningitis was excluded. Patients' demographic, social and clinical characteristics are presented in Table 1. Black African patients, altered consciousness, focal neurological deficits and symptom duration > 5 days were significantly more common in the TBM Group than in the Non-TBM Group. Furthermore, patients in the TBM Group presented significantly less often with headache and were encountered less frequently in the summer than patients in the Non-TBM Group. Table 2 comprises a detailed description of the TBM Group.

Serum 25-hydroxyvitamin D levels in tuberculous meningitis and non-tuberculous meningitis patients

A third of the study population ($n = 32$) had serum 25(OH)D levels < 50 nmol/L and 9.3% ($n = 9$) had serum 25(OH)D levels < 25 nmol/L. The mean concentration of serum 25(OH)D in the total study population was 72.9 nmol/L (± 36.6), with a statistically significant difference ($p = 0.001$) between TBM (mean 61.1 ± 27.8) and non-TBM (mean 84.3 ± 40.6) patients (Figure 1). Levels of 25(OH)D < 75 nmol/L were significantly more common in TBM patients ($n = 32$, 66.7%) than non-TBM patients ($n = 17$,

Table 2: Diagnostic characteristics of tuberculous meningitis patients

		TBM
		N (%) [IQR]
Diagnosis TBM[a]	Definitive	26 (54.2)
	Probable	16 (33.3)
	Possible	6 (12.5)
Drug susceptibility (n=27)	DS	17 (63.0)
	IMR	2 (7.4)
	Unknown	8 (29.6)
Signs and symptoms		
Symptoms suggestive of TB		11 (22.9)
TB contact in history (n=47)		24 (51.1)
TBM stage [b] (n=47)	I	10 (21.3)
	IIa	14 (29.8)
	IIb	13 (27.7)
	III	10 (21.3)
Focal motor deficit		13 (27.1)
Cranial nerve palsy		19 (39.6)
Evidence of BCG		47 (97.9)
TST, positive (n=25)		15 (60.0)
CSF		
Macroscopic clear appearance		42 (87.5)
Total cell count, cells/μL [c] (n=47)		93.0 [29.0–209.0]
Lymphocytes, cells/μL [c] (n=47)		86.0 [27.0–175.0]
Protein concentration, g/L [c] (n=41)		1.2 [0.9–2.0]
Glucose concentration, mmol/L [c] (n=40)		1.6 [1.0–2.5]
Cerebral imaging		
Hydrocephalus (n=47)		37 (78.7)
Basal meningeal enhancement (n=47)		34 (72.3)
Tuberculoma(s) (n=47)		9 (19.1)
Infarct (n=47)		15 (31.9)
Pre-contrast basal hyperdensity (n=45)		19 (42.2)

n = 48 unless otherwise stated; [c] Median and interquartile range (IQR) given;
[b] Refined TBM stage of van Toorn *et al*. [26]; [a] Diagnosis according to Marais *et al*. [20].
TST – tuberculin skin test; CSF – cerebrospinal fluid; DS – drug susceptible;
IMR – isoniazid monoresistant; BCG – bacillus Calmette–Guérin

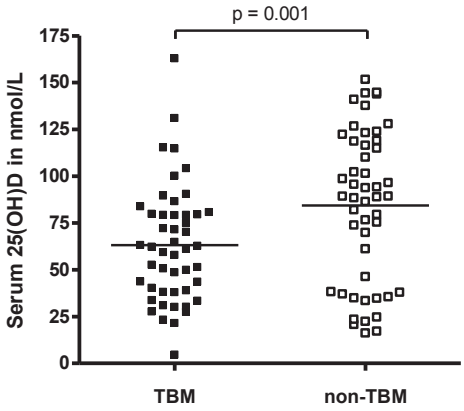

Figure 1: Serum 25-hydroxyvitamin D in tuberculous meningitis and non-tuberculous meningitis patients
Individual (black and white squares) and mean (black lines) 25-hydroxyvitamin D levels (nanomoles per litre) in tuberculous meningitis (TBM; n = 48; mean 61.1 ± 27.8) and non-TBM (n = 49; mean 84.3 ± 40.6) patients. The independent-sample t-test was used for statistical comparison.

34.7%; OR 3.77, 95% CI 1.63–8.72, $p < 0.01$). After adjustment for 'season of inclusion', this effect remained significant (OR 2.87, 95% CI 1.14–7.22, $p = 0.03$). No significant differences were evident for lower cut-off values (25(OH)D 50 nmol/L: OR 1.5, 95% CI 0.64–3.52, $p = 0.35$; 25(OH)D 25 nmol/L: OR 0.48, 95% CI 0.11–2.03, $p = 0.32$).

Serum 25-hydroxyvitamin D levels and concentrations of cathelicidin LL-37 and five other vitamin D-related biomarkers in cerebrospinal fluid

No significant correlation was apparent between serum 25(OH)D and cathelicidin LL-37 concentrations in CSF of the total study population ($\rho = -0.02$, $p = 0.85$). When the TBM and Non-TBM Groups were analysed separately, a significant correlation was evident in the Non-TBM Group (Non-TBM Group: $\rho = 0.345$, $p = 0.02$; TBM Group: $\rho = -0.122$, $p = 0.41$). Differences in median CSF cathelicidin LL-37 concentration for the three 25(OH)D cut-off values (75, 50 en 25 nmol/L) are illustrated in Figure 2. Statistically significant differences were evident in only the Non-TBM Group for cut-off values of 75 nmol/L ($p < 0.01$) and 50 nmol/L ($p = 0.01$).

Biomarker expression in the CSF of all study participants is outlined in Figure 3. Significant correlations were found between levels of cathelicidin LL-37 and IFN-γ ($\rho = 0.31$, $p < 0.01$), IP-10 ($\rho = 0.41$, $p < 0.01$), RANTES ($\rho = 0.21$, $p = 0.04$) and IL-13 ($\rho = 0.40$, $p < 0.01$), but not IL-15 ($\rho = 0.12$, $p = 0.26$).

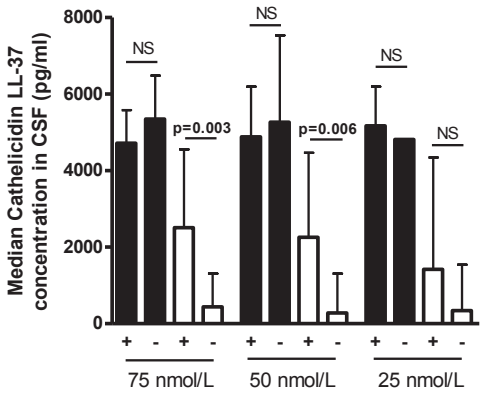

Figure 2: Differences in cerebrospinal fluid cathelicidin LL-37 concentrations in tuberculous meningitis and non-tuberculous meningitis patients for different 25-hydroxyvitamin D cut-off values
Median cathelicidin LL-37 concentrations on the *y*-axis are expressed as picograms per millilitre (pg/mL). On the *x*-axis, the black bars correspond to patients with tuberculous meningitis (TBM; $n = 48$) and the white bars correspond to non-TBM patients ($n = 49$). Three cut-off values for serum 25-hydroxyvitamin D were analysed (75 nmol/L, 50 nmol/L and 25 nmol/L). NS – not significant.

DISCUSSION

In this study, we demonstrated that low serum 25(OH)D levels are associated with TBM in a paediatric population with suspected meningitis. An association between low 25(OH)D levels and increased risk of active TB was demonstrated previously [8] and a reciprocal causal relationship between seasonal variation in vitamin D levels and the incidence of TB was suggested [27]. To date, no data are available for patients with TBM, except those showing an association between seasonal variation in the incidence of TBM and the duration of exposure to sunshine prior to disease manifestation [28]. Whether vitamin D levels affect susceptibility to tuberculosis infection or the progression of latent to active disease is unclear. Previous studies have illustrated that the role of serum 25(OH)D in *M. tuberculosis* related disease is to maintain the localized production of cathelicidin LL-37 following activation of monocytes by TLR ligands [10, 18]. Ex vivo, low levels of 25(OH)D (< 75 nmol/L) are associated with decreased *hCAP-18* mRNA expression by monocytes following immune challenges by TLR ligands, with improvement of the immune response after vitamin D supplementation [18]. Intriguingly, and in contrast to what we expected, we observed a correlation between serum 25(OH)D and CSF cathelicidin LL-37 levels in non-TBM patients with suspected meningitis but not in patients with TBM. Furthermore significant differences in CSF cathelicidin LL-37 concentration for different 25(OH)D cut-off values (50 and 75 nmol/L) were only found in the non-TBM

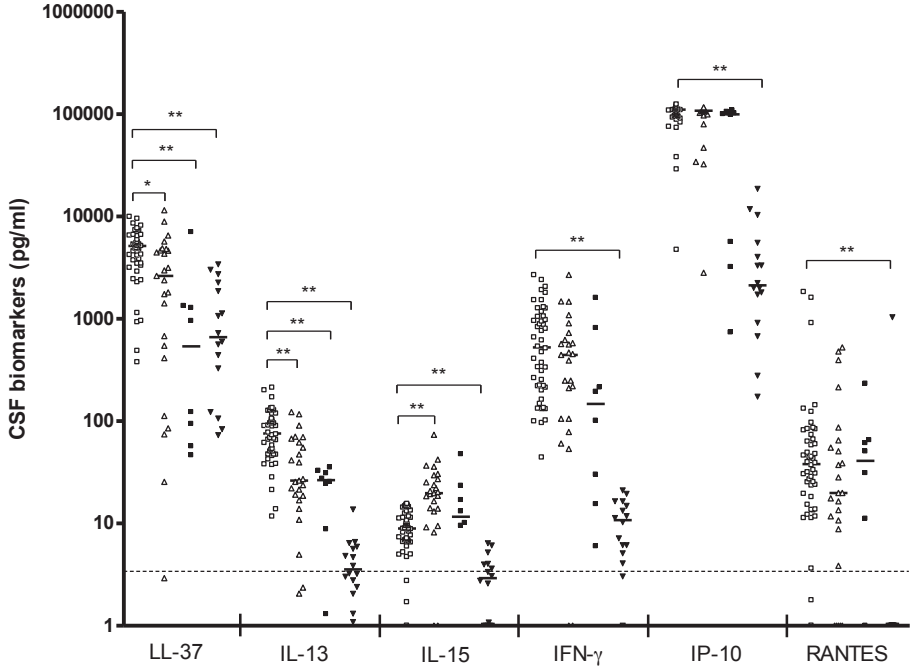

Figure 3: Cerebrospinal fluid biomarker expression levels in patients with signs and symptoms of meningitis

Individual and median (black lines) biomarker concentrations in the cerebrospinal fluid expressed as picograms per millilitre (pg/mL). Open squares (□) correspond to patients with tuberculous meningitis (TBM; *n* = 48); open triangles (△) correspond to patients with viral meningitis (*n* = 25); closed squares (■) correspond to patients with bacterial meningitis (*n* = 8); closed triangles (▼) correspond to patients without meningitis (*n* = 16). The dotted line represents the average limit of detection for all biomarkers. Statistical comparisons were made using pairwise comparison with Kruskal-Wallis Test (*p < 0.05 and ≥ 0.01; **p < 0.01). Only significant differences between the TBM group and the non-TBM subgroups are outlined in the figure.

group. Given that cathelicidin LL-37 production in monocytes seems to be dependent on both TLR ligands and 25(OH)D, the high levels of CSF cathelicidin LL-37 in a relative low 25(OH)D environment in patients with TBM, suggest a vigorous immune stimulation by *M. tuberculosis* specific TLR ligands that may disturb the correlation between serum 25(OH)D and CSF cathelicidin LL-37 in TBM patients.

It was demonstrated previously that pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α), IL-6, IL-1β and IFN-γ, show seasonal variation in healthy subjects and are related to serum vitamin D levels [29]. We studied correlations between biomarkers involved in the vitamin D-mediated human antimicrobial response and cathelicidin LL-37 levels in the CSF and found correlations between cathelicidin LL-37 and IL-13, IFN-γ, RANTES and IP-10, which emphasizes the impor-

tance of cathelicidin LL-37 in the innate immune response. Correlation between IL-13 and cathelicidin LL-37 levels in the CSF has not been described before, but IL-13 is known to regulate vitamin D metabolism by inducing cathelicidin LL-37 expression in bronchial epithelial cells. This is most likely a consequence of the upregulation of cytochrome P450 27B1 (25(OH)D 1α-hydroxylase), an enzyme that catalyses the hydroxylation of 1,25D [15]. IFN-γ, a well-known pro-inflammatory cytokine that protects against TB, also upregulates 25(OH)D 1α-hydroxylase and inhibits the induction of 25(OH)D 24-hydroxylase, leading to prolonged bioavailability of 1,25D [30, 31] and upregulation of the *hCAP-18* gene [32]. In epidermal keratinocytes, cathelicidin LL-37 modulates pro-inflammatory responses after TNF-α and/or IFN- γ stimulation, and influences the production of RANTES and IP-10 [33]. A correlation between the concentrations of cathelicidin LL-37 and RANTES or IP-10 in the CSF has not described before.

Our study has certain limitations. First, only a small part of the study population had severe vitamin D deficiency (< 25 nmol/L), which limited us to do subgroup analyses in this particular group. Second, a case definition based on consensus [20] was used for TBM diagnosis, with only 54% definite TBM cases. This could have caused misclassification bias. Third, the observational design of this study did not allow us to determine whether low 25(OH)D levels are a result of, or a risk factor for, the disease process. Although TBM is the most common cause of paediatric bacterial meningitis in the Western Cape of South Africa [2], the incidence is too low to perform a population-based cohort study to answer this question. Currently, the best explanation for low vitamin D levels in patients with active TB is that a fall in serum 25(OH)D concentration activates latent disease [34].

CONCLUSIONS

In this study, we demonstrated an association between low serum 25(OH)D levels and TBM diagnosis in a paediatric population of patients with suspected meningitis. In *M. tuberculosis* disease, vitamin D has been suggested to play a key role in maintaining localized production of cathelicidin LL-37, with suppression of its production in a vitamin D insufficient environment. CSF cathelicidin LL-37 concentrations were high in TBM patients but did not correlate with serum 25(OH)D levels. Strong positive correlations between several vitamin D-associated biomarkers (IFN-γ, RANTES, IP-10 and IL-13) and cathelicidin LL-37 levels in the CSF were found. Given that the balance between pro- and anti-inflammatory cytokines is thought to contribute to disease progression, seasonal variation in serum levels of 25(OH)D and related localized biomarkers could disturb this balance and activate latent disease.

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5 Vitamin D deficiency in native Dutch and first- and second-generation non-Western immigrants

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ABSTRACT

The aim of this study was to determine the prevalence of 25-hydroxyvitamin D (25(OH)D) deficiency in a hospital-based population of both native Dutch and non-Western immigrants and to investigate the influence of immigrant status on prevalence of vitamin D deficiency. A cross-sectional survey was conducted among 132 patients (1-18 years of age) visiting the paediatric outpatient department. Serum levels of 25(OH)D were measured using high-performance liquid chromatography. Cut-off levels of 30 nmol/l and 50 nmol/l for serum 25(OH)D were evaluated. One third of the patients had serum 25(OH)D levels below 30 nmol/l and half of the study population had serum levels below 50 nmol/l. Non-Western immigrants had an increased risk for vitamin D deficiency compared to their native Dutch peers (25(OHD) <30 nmol/l: p=0.03, OR 3.87 (95%CI 1.13 – 13.29); 25(OH)D <50 nmol/l: p= 0.02, OR 3.57 (95%CI 1.26 – 10.14)) with the highest risk for first-generation non-Western immigrants.

Conclusion – Vitamin D deficiency in the paediatric population is still a matter of concern in the Netherlands, in particular among first-generation non-Western immigrants. We therefore strongly recommend vitamin D supplementation for all non-Western immigrants, regardless of age, skin type or season. Healthcare staff who work with non-Western immigrants should be aware of the prevalence and implications of vitamin D deficiency.

INTRODUCTION

Approximately 1 billion people worldwide have low serum levels of vitamin D. The highest prevalence of vitamin D deficiency occurs in Asia and the Middle East [1]. Not only adults are at risk: vitamin D deficiency is also pandemic among the paediatric population [2].

Vitamin D is a generic term for a group of steroid hormones that are derived primarily from cutaneous photosynthesis. Dietary sources only contribute little to total serum levels of vitamin D [3]. In the intestine and bone, vitamin D interacts with the vitamin D receptor to both enhance intestinal absorption of calcium and mobilize osteoclastic activity [4]. Recently it has become increasingly appreciated that vitamin D has pleotropic effects with a key role in regulation of the human immune system, [4, 5] and low vitamin D serum concentrations associated with diseases such as tuberculosis, cancer, cardiovascular disease and multiple sclerosis [4,6-9].

The long-lived plasma metabolite 25-hydroxyvitamin D [25(OH)D] is a useful marker of vitamin D deficiency. It is produced in the liver and reflects the total amount of vitamin D obtained from both synthesis in the skin and dietary intake [10]. There is no general agreement about the adequate serum level of 25(OH)D but serum levels of 50 nmol/l cover requirements of at least 97.5% of the population between 1-70 year of age [11]. Serum concentrations below 30 nmol/l are often accompanied by elevated levels of parathyroid hormone and disturbances in calcium homeostasis and bone mineralization and therefore frequently considered as cut-off for vitamin D deficiency [12].

Non-Western populations who migrate to countries at higher latitudes are known to be at risk for vitamin D deficiency [25]. Contributory factors that are associated with vitamin D deficiency in immigrants are skin type, time spent outdoors, age, low socioeconomic status, wearing of body-covering clothes, and a diet low in fish and dairy products [1].

In Amsterdam, The Netherlands, half of the population, and two-thirds of the paediatric population, are of non-Dutch descent; the largest groups within this subset of the population originate from Morocco, Turkey, and Surinam. Up to two-thirds of the adult non-Western immigrant population in the Netherlands are vitamin D deficient [13, 14]. Two studies showed a high prevalence of vitamin D deficiency in immigrant children and adolescents in Western Europe, [15, 16] but little is known about the differences in vitamin D status between first- and second-generation immigrants. The Dutch Health Council recommends that children younger than 4 years of age should take 10 micrograms vitamin D supplementation daily [12]. Thereafter vitamin D supplementation is only recommended for those with a dark skin or with limited sunlight exposure, despite the presumed high prevalence of vitamin D

deficiency in immigrants [12]. In the study reported herein, we aimed to determine the prevalence of vitamin D deficiency in a hospital-based paediatric population of both native Dutch and non-Western immigrants and investigated the influence of immigrant status on prevalence of vitamin D deficiency.

PATIENTS AND METHODS

A cross-sectional survey was conducted among patients aged 1–18 years, who visited the paediatric outpatient department of a district teaching hospital in the Western region of Amsterdam. Patients and/or their carers were asked to participate in the study when venous puncture had to be performed for any clinical reason. The following patients were excluded from the study: those who originated from Western countries outside the Netherlands, those who had specific conditions (e.g., chronic kidney disease, parathyroid disease, inflammatory bowel disease, celiac disease, or severe immunodeficiency) or used specific medication that are known to influence vitamin D levels (e.g., corticosteroids, antiepileptic drugs, or calcium supplements). The attending paediatrician or paediatric registrar obtained information about ethnic background, skin type and dietary habits by administering a short questionnaire. Skin type was divided into three groups, on the basis of the Fitzpatrick Skin Phototype Classification [17]: Skin type 1 (white) comprised Fitzpatrick skin types 1 and 2; Skin type 2 (coloured) comprised Fitzpatrick skin types 3 and 4; Skin type 3 (black) comprised Fitzpatrick skin types 5 and 6. Dietary habits were evaluated with questions about intake of vitamin D containing products like dairy products, fatty fish and meat. Standard deviations (SD) of body mass index (BMI) values were based on the World Health Organization (WHO) Child Growth Standards, and were divided into three groups (normal weight: –1 SD to 1 SD; overweight: 1 to 2 SD; adiposity: >2 SD) [18, 19].

Non-Western immigrants included all persons with a Turkish, African, Asian, or Latin American background. Patients with a Japanese or Indonesian background were classified as Western immigrants in the Netherlands, and therefore excluded from the study. The patients in the study were subdivided into three groups: native Dutch, for whom the parents and patients were born in the Netherlands; patients who were born outside the Netherlands and had at least one parent who originated from outside the Netherlands (first-generation immigrants); and patients who were born in the Netherlands and had at least one parent who was a first-generation immigrant (second-generation immigrants).

Serum levels of 25(OH)D were measured using high-performance liquid chromatography followed by ultraviolet detection, in accordance with the manufacturer's

protocol (Chromsystems Instruments & Chemicals GmbH, Munich, Germany). As there is a lot of discussion in the literature about the adequacy of vitamin D status cut-off values for serum 25(OH)D of 30 nmol/l and 50 nmol/l were analysed.

Ethical approval

The study was conducted according to the ethical guidelines and principles of the International Declaration of Helsinki. Ethical approval was obtained from the Medical Ethics Committee of the Sint Lucas Andreas Hospital, Amsterdam. Written informed consent was obtained from all patients or their caregivers.

Statistical analyses

The Statistical Package for the Social Sciences version 19.0 for MAC (IBM®) was used for the statistical analyses. Differences between native Dutch and non-Western immigrants were analysed using Chi-square test or Fisher's Exact test for frequencies and Mann–Whitney U test for continuous variables. Pairwise comparisons of non-parametric data have been performed using the Kurskal-Wallis test. Second, potential predictors for vitamin D deficiency were analysed using univariable logistic regression analyses. Odds ratios with 95% confidence intervals (95%CI) were calculated to measure the effect size. Potential confounders that led to a change in the coefficient of more than 10% and confounding factors previously described in literature, like season and skin type, were added to the model. In all analyses, $P \leq 0.05$ was considered statistically significant.

RESULTS

A total of 132 patients were included in the study between January and June 2011. The demographic characteristics are outlined in Table 1. One-quarter of the total study population had an indigenous Dutch background (n = 35). Of the non-Western immigrants, 80 belonged to the second-generation and 17 to the first-generation immigrants. A statistically significant difference between the native Dutch group and the group of non-Western immigrants was observed for skin type ($p < 0.001$) and intake of fatty fish ($p = 0.03$).

Table 1. Demographic characteristics of native Dutch and non-Western immigrant children

	Native Dutch	Immigrant	
Demographic characteristic	N	N	*P*
Total number	35	97	
Sex			0.89
Male	16	43	
Female	19	54	
Age, months*	80.0 [13.2 – 198.0]	104.7 [12.5 – 212.0]	0.15
Age			0.57
< 4 years	6	21	
≥ 4 years	29	76	
BMI (n = 123)			0.35
Normal	28	62	
Overweight	4	20	
Obese	2	7	
Dietary habits# (n = 131)			
Fatty fish	15	62	0.03**
No fatty fish	20	34	
Dairy products	33	89	1.00**
No dairy products	2	7	
Meat	33	88	1.00**
No meat	2	8	
Skin type (n = 125)			<0.001
I	28	29	
II	5	55	
III	0	8	
Vitamin D suppl. (n = 121)			0.60
Yes	7	15	
No	26	73	
Season of inclusion			0.07
I: January - March	19	69	
II: April - June	16	28	

* Mann Whitney test used, data expressed as median and range; ** Fisher's Exact test used. In all other cases Chi-square test was used for frequencies. # Dietary habits: intake of fatty fish, dairy products and/or meat for at least once a week.

Immigrant status and vitamin D deficiency

The median level of serum 25(OH)D in the total study population was 49.0 nmol/l [range 6.0–264.0] with a statistically significant difference (P <0.001) between non-Western immigrants (38.0 nmol/l [range: 9.0–113.0]) and native Dutch (61.0 nmol/l [IQR 6.0–264.0]). First-generation non-Western immigrants had the lowest serum 25(OH)D levels (median 31.0 nmol/l [range: 9.0 – 111.0]) (Figure 1).

Univariable logistic regression analysis showed an increased risk for vitamin D deficiency in non-Western immigrants (first- and second-generation) and in those included in the winter season (Table 2). Patients below the age of 4 and those who got vitamin D supplementation (only for serum 25(OH)D <30 nmol/l) had a decreased risk for vitamin D deficiency (Table 2). When results were adjusted for age, season of inclusion and skin type (last one only for 25(OH)D <50 nmol/l), non-Western immigrants still had an increased risk for vitamin D deficiency compared to the native Dutch group (25(OH)D <30 nmol/l: p=0.03, OR 3.87 (95%CI 1.13 – 13.29); 25(OH) D <50 nmol/l: p= 0.02, OR 3.57 (95%CI 1.26 – 10.14)). Evaluation of immigration status on the risk for vitamin D deficiency showed that first-generation non-Western immigrants had the highest risk for vitamin D deficiency (25(OH)D <30 nmol/l: p=0.009, OR 6.63 (95%CI 1.62 – 27.13); 25(OH)D <50 nmol/l: p=0.006, OR 7.11 (95%CI 1.75 – 28.94)) compared to the native Dutch group (data adjusted for age, and also

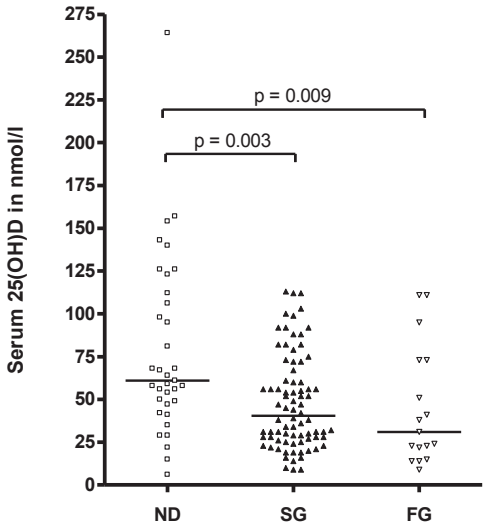

Figure 1

Individual and median 25(OH)D levels (nmol/l) in native Dutch (ND) and first- (FG) and second-generation (SG) non-Western immigrants. Pairwise statistical comparisons were performed with the non-parametric Kurskal-Wallis test. Exclusion of the outlier in the native Dutch group gave comparable results (p = 0.006 and p = 0.012 respectively).

adjusted for season of inclusion for 25(OH)D <50 nmol/l). For second-generation non-Western immigrants a lower, but still significant risk was found (25(OH)D <30 nmol/l: p=0.03, OR 3.21 (95%CI 1.10 – 9.43); 25(OHD) <50 nmol/l: p=0.007, OR 3.65

Table 2. Univariable logistic regression analysis for serum 25(OH)D <30 nmol/l and <50 nmol/l

Demographic characteristic		**25(OH)D <30 nmol/l**			**25 (OH)D <50 nmol/l**	
	Total N	N	OR (95% CI)	*P*	N	OR (95% CI)	*P*
Sex							
Male	59	13	1.00		28	1.00	
Female	73	26	1.96 (0.90 – 4.27)	0.091	39	1.27 (0.64 – 2.50)	0.500
Age							
< 4 years	27	2	0.15 (0.03 – 0.66)	0.012	5	0.16 (0.06 – 0.45)	0.001
≥ 4 years	105	37	1.00		62	1.00	
Immigrant status							
Native Dutch	35	5	1.00		10	1.00	
Second generation	80	26	2.89 (1.01 – 8.31)	0.049	46	3.38 (1.44 – 7.97)	0.005
First generation	17	8	5.33 (1.39 – 20.43)	0.015	11	4.58 (1.33 – 15.77)	0.016
BMI (n=123)							
Normal	90	25	1.00		44	1.00	
Overweight	24	9	1.56 (0.61 – 4.02)	0.357	14	1.46 (0.59 – 3.64)	0.412
Obese	9	4	2.08 (0.52 – 8.38)	0.303	5	1.31 (0.33 – 5.19)	0.704
Dietary habits (n=131)#							
Fatty fish	77	23	1.01 (0.47 – 2.12)	0.976	42	1.39 (0.69 – 2.80)	0.353
No fatty fish	54	16	1.00		25	1.00	
Dairy products	122	36	0.84 (0.20 – 3.53)	0.809	62	0.83 (0.21 – 3.23)	0.784
No dairy products	9	3	1.00		5	1.00	
Meat	121	37	1.76 (0.36 – 8.70)	0.487	62	1.05 (0.29 – 3.82)	0.940
No meat	10	2	1.00		5	1.00	
Skin type (n=125)							
I	57	12	1.00		23	1.00	
II	60	21	2.02 (0.88 – 4.63)	0.097	34	1.93 (0.93 – 4.03)	0.079
III	8	3	2.25 (0.47 – 10.78)	0.310	4	1.48 (0.36 – 6.52)	0.606
Vitamin D suppl. (n=121)							
Yes	22	1	0.09 (0.01 – 0.68)	0.019	8	0.52 (0.20 – 1.34)	0.175
No	99	35	1.00		52	1.00	
Season of inclusion							
I January – March	88	32	3.03 (1.20 – 7.69)	0.018	52	2.79 (1.31 – 5.94)	0.008
II April – June	44	7	1.00		15	1.00	

Dietary habits: intake of fatty fish, dairy products and/or meat for at least once a week

(95%CI 1.42 – 9.38)). However, no statistically significant increased risk for vitamin D deficiency was found when first- and second-generation non-Western immigrants were compared.

Dutch Health Council recommendations

Of the patients below the age of 4 (n=23), 9 got vitamin D supplementation compared to 13 in the group of patients older than 4 year of age (n=98) (p=0.006, OR 4.20 (95%CI 1.52 – 11.67)). None of the patients with a dark skin (skin type III) received supplemental vitamin D.

DISCUSSION

To the best of our knowledge, this is the first study in which the vitamin D status of native Dutch patients and of first- and second-generation non-Western immigrants has been investigated. One third of our study population had serum 25(OH)D levels below 30 nmol/l and half of the patients had serum levels below 50 nmol/l. Non-Western immigrants and in particular the first generation immigrants proved to have an increased risk for vitamin D deficiency compared to their native Dutch peers. As mentioned before, the Dutch Health Council recommends that all children should take vitamin D supplementation up till the age of 4 years [12]. Thereafter vitamin D supplementation is advised to those with a dark skin or with diminished sunlight exposure. Contrary to these recommendations, only a minority of patients younger than 4 years of age and none of the older patients with a dark skin type received vitamin D supplementation. Although the present study included a small number of patients, the implementation of the Dutch Health Council recommendations in clinical practice seems to be reason for concern. Given that we found a clear difference in vitamin D status between the native Dutch group and non-Western immigrants, we strongly recommend supplementation of vitamin D for all non-Western immigrants in the Netherlands irrespective of age or skin type, and in particular first-generation immigrants.

Various factors contribute to the increased risk for vitamin D deficiency among non-Western immigrants [1]. Skin type is one of those factors. Cutaneous photosynthesis of vitamin D depends on the dose of solar Ultraviolet-B (UVB) radiation, skin type, and age [20]. During the winter months in Northern European countries, solar UVB radiation declines and can lead to insufficient vitamin D synthesis in particular in the dark pigmented individuals [21]. Two third of the study population was investigated during winter months (January to March). They had statistically significant more vitamin D deficiency compared to those included between April and June.

Although results were adjusted for age, skin type and season of inclusion, differences in vitamin D status between non-Western immigrants and the group of native Dutch patients remained.

The contribution of human diet to adequate serum vitamin D levels is known to be relatively small [3, 20]. However, if cutaneous photosynthesis is limited as a result of season or cultural habits (indoor living, wearing a veil), dietary habits may play a more prominent role in the prevalence of vitamin D deficiency among immigrants. In the past, research in children of asylum seekers in the Netherlands showed a low dietary vitamin D intake compared with that of native Dutch children [22]. In our study population non-Western immigrants ate significant more fatty fish compared to the native Dutch group, but no confounding effects on prevalence of vitamin D deficiency between the two groups were seen.

Although 25(OH)D deficiency among non-Western immigrants is a well-known phenomenon, an association between immigrant status and risk of vitamin D deficiency has not been described before. One can only speculate what reasons could explain this. Acculturation, the process that occurs when members of a minority group adopt the cultural patterns of the host country in time might have contributed to this finding but further research is necessary [23, 24].

We accept that there are limitations to our study. Given that it was a cross-sectional hospital-based study, there is a risk of selection bias. Fortunately the distribution of ethnicities in the study population was similar to that of the population of the catchment area of the hospital [25]. Even so, the vitamin D status of the native Dutch group was comparable with previously published results [26]. The small sample size and the fact that no validated questionnaire was used to assess dietary intake could have biased the outcome. Despite these limitations, the differences in prevalence of 25(OH)D deficiency between the native Dutch group and non-Western immigrants in the studied population were obvious.

CONCLUSIONS

Vitamin D deficiency in the paediatric population is still a matter of concern in the Netherlands, in particular among first-generation non-Western immigrants. According to the Dutch Health Council no recommendations for vitamin D supplementation are given for this group after the age of 4 years except for those with a dark skin and limited sunlight exposure. Based on our results we strongly recommend vitamin D supplementation for all non-Western immigrants, regardless of their age, skin type or season. Healthcare staff who work with immigrants should be aware of the prevalence and implications of vitamin D deficiency.

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Part II Early diagnosis in childhood tuberculous meningitis and clinical outcome

6 Lipoarabinomannan enzyme-linked immunosorbent assay for early diagnosis of childhood tuberculous meningitis

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ABSTRACT

Setting -Tuberculous meningitis is a severe complication of tuberculosis and predominantly affecting young children. Early treatment initiation is important to avoid associated morbidity and mortality, emphasizing the importance of early diagnosis. Among the most promising new methods for diagnosing tuberculosis are antigen-detection assays based on the detection of lipoarabinomannan.

Design - A cross-sectional study, in which urine samples from paediatric patients with suspected tuberculous meningitis attending Tygerberg Children's Hospital, Cape Town, South Africa were tested for lipoarabinomannan.

Objective: To evaluate the diagnostic value of a commercial, antigen-capture enzyme-linked immunosorbent assay test based on the detection of lipoarabinomannan in urine for the early diagnosis of tuberculous meningitis in children.

Results - Complete data were available for 50 of 56 patients with suspected tuberculous meningitis. Tuberculous meningitis was diagnosed in 21 (42%) and confidently excluded in 29 (58%) patients. The lipoarabinomannan enzyme-linked immunosorbent assay had a sensitivity of 4.8% and specificity of 93.1%. Serial measurements in the first 2 weeks after treatment initiation did not improve test performance.

Conclusion - We showed that urinary lipoarabinomannan detection was of little value for the diagnosis of tuberculous meningitis in a cohort of paediatric patients with suspected tuberculous meningitis.

INTRODUCTION

Tuberculous meningitis (TBM) is a severe complication of tuberculosis (TB) and predominantly affecting young children [1]. Early treatment initiation has the most significant impact on morbidity, mortality and healthcare costs, emphasizing the importance of early diagnosis [2, 3]. Mycobacterial culture of cerebrospinal fluid (CSF) is the laboratory standard for diagnosis but is not widely available in resource-constrained settings. Mycobacterial culture can take several weeks to yield results and demonstrates a sensitivity of just 12% in paediatric patients [4, 5]. Polymerase chain reaction-based diagnostic tests are still undergoing assessment and are not yet suitable for widespread use in resource-poor countries [6]. Diagnostic tests for acid-fast bacilli using Ziehl–Neelsen staining are rapid and inexpensive but exhibit a low sensitivity, ranging from 10–20% [6].

The lack of sensitive methods for early TBM diagnosis, rather than late presentation or inaccessibility to medical care, is the most common cause for delayed diagnosis [7]. Many attempts have been made to develop simplified tests for TB but their diagnostic power remains poor. A sensitive, specific, simple test format, such as the enzyme-linked immunosorbent assay (ELISA), would provide a major advantage. Among the most promising new methods for diagnosing TB are antigen-detection assays based on the detection of lipoarabinomannan (LAM), a *Mycobacterium*-specific lipopolysaccharide of the bacillus cell wall [8]. LAM is released from metabolically active and disintegrating mycobacteria into the bloodstream [9]. Urinary excretion of LAM is considered independent of the anatomical location of the infection, although a strong association with mycobacteriuria has been demonstrated [10, 11]. Urinary tests are attractive because urine is an easily obtainable biological fluid [9, 12]. Although tests for urinary LAM demonstrated inadequate sensitivity for the diagnosis of active TB in unselected cohorts, [13] it is worthwhile to study their performance in childhood TBM. To the best of our knowledge, no data exist on LAM in the urine samples of paediatric patients with TBM.

In this study, we evaluated the sensitivity and specificity of a commercial LAM ELISA test using urine samples from children with suspected TBM. In patients with pulmonary TB, the majority of mycobacteria are killed in the first few days after treatment initiation [14, 15]. These early bactericidal effects could be reflected in the presence of urinary LAM.16 After an initial 2 weeks of TB treatment, urinary LAM steadily declines [11]. With this in mind, we investigated the optimum time of antigen testing for urinary LAM.

STUDY POPULATION AND METHODS

Study population and case definition

A cross-sectional study was conducted among children aged between 3 months and 13 years with symptoms and signs suggestive of meningitis attending Tygerberg Children's Hospital, Cape Town, South Africa between June 2011 and February 2012. Symptoms and signs of meningitis included one or more of the following: headache, irritability, vomiting, fever, neck stiffness, convulsions, focal neurological deficits, altered consciousness, and lethargy. Patients with a clinical picture suggestive of meningitis entered the study after lumbar puncture or brain imaging. Afterwards they were classified according to the diagnostic criteria of Marais *et al*. [17].

TBM was classified as Definite when acid-fast bacilli were evident in the CSF, *Mycobacterium tuberculosis* (*M. tuberculosis*) was cultured from the CSF, or *M. tuberculosis* was detected by a reliable molecular method in the CSF in a patient with symptoms or signs suggestive of the disease. In this study auramine-staining technique was used for direct fluorescence microscopy, BACTEC MGIT 960 (Becton Dickinson Diagnostic Systems, USA) method for culture and two commercial nucleic acid amplification tests, the GenoType MTBDRplus (Hain Life Science, Nehren, Germany) and GeneXpert MTB/RIF (Cepheid, Sunnyvale, USA) as molecular methods. All tests were used according to manufacturers guidelines. TBM was classified as Probable when patients had a diagnostic score ≥12 when imaging was available and ≥10 when imaging was unavailable. TBM was classified as Possible when a patient had a diagnostic score of 6–11 when imaging was available and 6–9 when imaging was unavailable [17]. Patients were classified as Non-TBM when an alternative diagnosis was established.

Ethics

The study was conducted according to the ethical guidelines and principles of the International Declaration of Helsinki and the South African Guidelines for Good Clinical Practice. Ethical approval was obtained from the Stellenbosch University Human Research Ethics Committee. Written informed consent was obtained from all patients or their caregivers.

Lipoarabinomannan measurement

From each patient with suspected meningitis, ≥5 ml urine was collected before treatment initiation (T0) and on days 1, 2, 3, 7, 11 and 15 (T1–T15) after treatment initiation. First-voided urine was collected from toilet-trained children and from younger children urine was collected with a urine collection bag. Collected urine specimens were stored at −80°C within 1 hour of collection.

LAM was measured using the Clearview TB ELISA Test (Alere Health BV, Tilburg, The Netherlands) according to the manufacturer's instructions, except that samples were frozen directly after collection before batch testing. Urine was heated for 30 minutes at 100°C and centrifuged at 10 000 rpm for 15 minutes. Capture antibodies were coated on to the surface of each well of 96-well plates, seeded with 100 µl of supernatant and developed according to the manufacturer's instructions. Wash procedure was practised at all stages manually. Optical densities (ODs) were read at 450 nm by a trained technician blinded to patient details. Duplicate positive and negative controls were processed for each batch. The average OD of the duplicate negative controls plus 0.1 OD units was considered the cut-off: specimens with ODs above the cut-off were considered positive for urinary LAM, whereas specimens with ODs below the cut-off were considered negative. A patient was considered LAM positive if at least one sample from any time point was positive.

Statistical analyses

Statistical analyses were conducted using SPSS version 19 (SPSS Inc., Chicago, IL, USA). Differences in baseline characteristics between the TBM and Non-TBM Groups were analysed using univariable logistic regression analyses. Odds ratios with 95% confidence intervals (95%CI) were calculated to measure the effect size. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the LAM ELISA test were calculated. Diagnostic scores of the Probable and Definite TBM Groups were compared using an independent t-test. In the TBM Group, serial urine samples of individual patients were analysed using the paired sample t-test. In all analyses, a p-value ≤0.05 was considered statistically significant.

RESULTS

Baseline characteristics

Complete data were available for 50 of 56 children with suspected meningitis. The caretakers of two patients refused to participate (3.5%) and for four patients (7.1%) insufficient urine was collected. Of the 50 patients included, 21 (42%) were classified as TBM and 29 (58%) as Non-TBM. The Non-TBM Group consisted of nine cases of viral meningitis, seven of bacterial meningitis and a heterogeneous group of 13 non-meningitis cases (e.g. febrile convulsion, complex seizure, urinary tract infection and acute gastroenteritis). Their baseline characteristics and presenting symptoms are outlined in Table 1. Symptom duration of >5 days was more common in the TBM Group than the Non-TBM Group. The other presenting symptoms did not significantly differ between the groups.

Table 1 – Baseline patient characteristics

Characteristic	TBM	Non-TBM		
	N (%)	N (%)	OR (95%CI)	P
Total number of patients	21 (42.0)	29 (58.0)		
Age in months (Mean ± SD)	46.0 ± 35.4	54.5 ± 43.4		0.467
Sex, male	11 (52.4)	16 (55.2)	0.89 (0.29 – 2.76)	0.845
HIV-infected (n=20; n=28)	1 (5.0)	2 (7.1)	0.68 (0.06 – 8.11)	0.764
Race (n=21; n=27): *				
Black	5 (23.8)	7 (24.1)	0	
Mixed ancestry	16 (76.2)	20 (69.0)	1.12 (0.30 – 4.20)	0.867
Presenting symptoms:**				
Fever	19 (90.5)	23 (79.3)	2.48 (0.45 – 13.73)	0.299
Headache	6 (28.6)	15 (51.7)	0.37 (0.11 – 1.23)	0.106
Convulsions	4 (19.0)	10 (34.5)	0.48 (0.12 – 1.69)	0.236
Vomiting	9 (42.9)	18 (62.1)	0.46 (0.15 – 1.44)	0.181
Focal neurological deficit	6 (28.6)	2 (6.9)	5.40 (0.97 – 30.17)	0.055
Cranial nerve palsy	5 (23.8)	4 (13.8)	1.95 (0.46 – 8.38)	0.368
Change in behaviour†	12 (57.1)	12 (41.4)	1.53 (0.54 – 4.37)	0.427
Neck stiffness	7 (33.3)	7 (24.1)	1.50 (0.43 – 5.2)	0.524
Altered consciousness	5 (23.8)	7 (24.1)	0.98 (0.26 – 3.66)	0.979
Symptom duration > 5 days	18 (85.7)	8 (27.6)	15.75 (3.63 – 68.41)	<0.001

TBM=tuberculous meningitis; HIV= human immunodeficiency virus.
* Race: Due to the limited number of white patients (2 in the Non-TBM Group and 0 in the TBM Group) these patients were excluded for regression analysis. **Presenting symptoms: more than one symptom seen in most cases; †change in behaviour: irritability and lethargy were taken together.

According to the TBM criteria of Marais *et al.*, [17] 17 patients were classified as Probable TBM and 4 as Definite TBM. None of the patients met the criteria for Possible TBM. All patients with TBM received the full treatment course. Apart from the confirmation of *M. tuberculosis* in the CSF via microbiological identification or a positive commercial nucleic acid amplification test, diagnostic scores between the Definite and Probable TBM Groups did not significantly differ (the mean diagnostic score was 15.0 for the Definite Group versus 14.4 for the Probable Group; p=0.71). Therefore, patients in both groups were combined to form the TBM Group. Within the TBM Group, 10 patients had signs of 'tuberculosis elsewhere' confirmed by either Chest X-ray (CXR), gastric washing or sputum culture. The clinical characteristics of the TBM Group are presented in Table 2.

Table 2 – Clinical characteristics of patients with tuberculous meningitis

Clinical characteristics		TBM
		N (%) [IQR]
Diagnosis TBM		21
Definite		4 (19.0)
Probable		17 (81.0)
Possible		0 (0)
History and clinical signs		
Symptoms suggestive of TB†		10 (47.6)
TB contact in history#		12 (57.1)
TBM-stage*	I	6 (28.6)
	IIa	7 (33.3)
	IIb	3 (14.3)
	III	5 (23.8)
GCS (median; IQR)		13 [9.5-15.0]
TST positive (n=15)		12 (80.0)
Focal neurological deficit (excluding cranial nerve palsies)		6 (28.6)
Cranial nerve palsy		5 (23.8)
Altered consciousness		14 (66.7)
Evidence of BCG		18 (85.7)
CSF		
Clear appearance		20 (95.2)
Total cell-count - cells/μL (median; IQR)		95.0 [26.5 – 195.5]
Lymphocytes - cells/μL (median; IQR)		81.0 [23.0 – 181.5]
Protein concentration - g/L (median; IQR)		1.2 [0.4 – 2.0]
Glucose concentration - mmol/L (mean; SD)		2.1 (1.3)
Cerebral imaging (n=19)		
Hydrocephalus		12 (63.2)
Basal meningeal enhancement		13 (68.4)
Tuberculoma		6 (31.6)
Infarct		7 (36.8)
Pre-contrast basal hyperdensity		7 (36.8)
Evidence of tuberculosis elsewhere** (n=21)		10 (47.6)
Chest radiograph suggestive for (n=21):		
	Pulmonary TB	6 (28.6)
	Miliary TB	0
Positive TB culture elsewhere*** (n=20)		5 (25.0)

TBM=tuberculous meningitis; m=male; CSF=cerebral spinal fluid; TB=tuberculosis; PCR=polymerase chain reaction; CNS=central nervous system; IQR=interquartile range; BCG=Bacillus Calmette-Guérin; TST=Tuberculin Skin Test. †Symptoms suggestive of tuberculosis consisted one or more of the following: weight loss or poor weight gain, night sweats, or persistent cough for more than 2 weeks. #History of recent close contact with an individual with pulmonary tuberculosis or a positive TST or Interferon-Gamma Release Assay (IGRA). *Tuberculous meningitis stage is based on the 'refined' British Medical Research Council scale [18]; Stage 1: Glasgow Coma Scale (GCS) of 15, without focal neurological deficits; Stage 2a: GCS of 15, with focal neurological deficits, or GCS of 13–14, with or without focal neurological deficits; Stage 2b: GCS of 10–12, with or without focal neurological deficits; Stage 3: GCS <10, with or without focal neurological deficits.**Patient had evidence of TB elsewhere in case culture (sputum and/or gastric washing) and/or CXR was positive for TB. ***Cultures from sputum and/or gastric washing.

Lipoarabinomannan antigen test sensitivity and specificity

A total of 21 TBM and 29 Non-TBM patients were tested for urinary LAM. One patient from the TBM Group and two from the Non-TBM Group tested positive for LAM, equating to a sensitivity of 4.8% and a specificity of 93.1%. The NPV and PPV were 0.33 and 0.57, respectively. A true positive urine sample was obtained from an 11-month old human immunodeficiency virus (HIV)-infected patient on day 1 after treatment initiation (T1). The gastric washing culture was positive for *M. tuberculosis* and the CXR revealed hilar lymphadenopathy. Repeated testing of urine from the same patient over the subsequent 2 weeks did not yield a positive result. Of the two patients who exhibited false positive results in the Non-TBM Group, one tested positive for LAM on day 0 (T0) and the other on day 2 (T2). Repeated testing yielded only negative results. Both patients were HIV-negative and had no signs or symptoms of 'tuberculosis elsewhere'.

Serial urine samples

To evaluate the optimum time for LAM detection, we analysed serial urine samples collected from patients in the TBM Group on days 0, 1, 2, 3, 7, 11 and 15. As previously mentioned, the only true positive result came from a sample collected on day 1 after treatment initiation. Serial ODs from individual patients are depicted in Figure 1. ODs from paired samples were compared over time but no significant increase or decrease in mean OD was found. The magnitude of changes in OD is presented in Table 3.

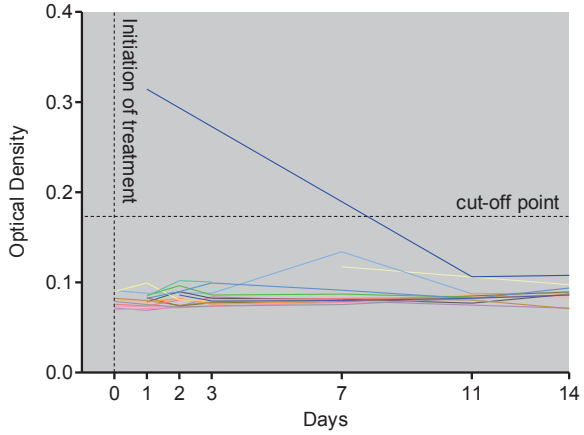

Figure 1

Serial optical densities as a result of lipoarabinomannan antigen in urine samples from individual patients (coloured lines) visualized over time. The x-axis shows the time-points (T0, T1, T2, T3, T7, T11 and T15) of urine collection. Urine was not collected from all patients at all time-points. The y-axis shows optical densities. The dotted horizontal line is the cut-off point of the Clearview TB ELISA Test (Alere Health BV, Tilburg, The Netherlands) for positive test results (0.1 OD units above the negative control). The vertical dotted line represents treatment initiation.

Table 3 – Paired sample t-test analysis of differences in mean optical density

Pairs	N	Mean difference in OD	95% CI	p-value
T0 - T1	8	0.0008	-0.0030 – 0.0045	0.647
T0 - T2	7	0.0028	-0.0022 – 0.0078	0.219
T0 - T3	6	0.0007	-0.0064 – 0.0077	0.817
T0 - T7	6	-0.0054	-0.0254 – 0.0146	0.518
T0 - T11	6	-0.0039	-0.0103 – 0.0025	0.177
T0 - T15	5	-0.0007	-0.0130 – 0.0116	0.882
T1 - T2	14	-0.0020	-0.0074 – 0.0034	0.434
T1 - T3	12	-0.0049	-0.0106 – 0.0008	0.084
T1 - T7	11	-0.0042	-0.0147 – 0.0063	0.396
T1 - T11	11	0.0156	-0.0273 – 0.0586	0.436
T1 - T15	10	0.0159	-0.0323 – 0.0640	0.475
T2 - T3	13	-0.0009	-0.0062 – 0.0043	0.709
T2 - T7	12	-0.0033	-0.0125 – 0.0058	0.440
T2 - T11	10	0.0010	-0.0048 – 0.0068	0.706
T2 - T15	9	-0.0018	-0.0063 – 0.0027	0.374
T3 - T7	12	-0.0036	-0.0127 – 0.0056	0.407
T3 - T11	9	0.0021	-0.0052 – 0.0094	0.534
T3 - T15	9	-0.0016	-0.0081 – 0.0049	0.583
T7 - T11	9	0.0057	-0.0068 – 0.0181	0.325
T7 - T15	9	0.0046	-0.0094 – 0.0186	0.469
T11 - T15	8	-0.0022	-0.0080 – 0.0035	0.385

OD=Optial density; CI=Confidence Interval

DISCUSSION

We found that the Clearview TB ELISA test had a poor sensitivity of 4.8% and a specificity of 93.1%. After treatment initiation, OD did not significantly change over time and, therefore, an optimum time for urine testing could not be determined. Recently, a multidisciplinary group of TB experts proposed the essential criteria for a point-of-care test [19]. For extrapulmonary TB, the test should provide a sensitivity of 80% (60% in the Probable Group) and a specificity of 95%. From this perspective, the assay tested seems to be of little clinical value for the diagnosis of TBM in the urine samples of unselected paediatric patients with suspected TBM. To the best of our knowledge, no previous studies examined urinary LAM antigen tests in paediatric or adult patients with suspected TBM. Two studies [20, 21] tested the Clearview TB ELISA test on CSF samples from adults with suspected TBM, identifying a sensitivity of 31–64% and a specificity of 69–94%. For the urine samples of patients with pul-

monary TB, a test sensitivity of 14% (4–38%) in HIV-uninfected individuals and 47% (26–68%) in HIV-infected patients was found, with a specificity >96% [22].

The Clearview TB ELISA test uses antibodies specific to LAM, a lipopolysaccharide present in the *M. tuberculosis* cell wall. Systemically-released, unbound LAM molecules (19 kDa) can pass freely through the glomerular membrane, resulting in a LAM-positive urine sample. However, systemically released LAM can bind to LAM antibodies to form large immune complexes with a limited capacity to pass through the glomerular membrane, resulting in a LAM-negative urine sample [11]. Because of their immunocompromised state, patients with HIV have a higher mycobacterial load4 and a decreased capacity for immune complex formation, [23] both of which result in increased levels of urinary LAM [13]. Wood *et al*. [11] showed that TB involving the renal tract, with *M. tuberculosis* in the urine, was present in almost half of LAM-positive patients. Therefore, the release of LAM into the urine directly from the renal tract could contribute to an increased test sensitivity in HIV co-infected patients. Given the inverse relationship between CD4+ T-cell count and urinary LAM sensitivity, [8] the low number of HIV-infected TBM patients in our study could have contributed to the low sensitivity observed. Remarkably, the only true positive urine sample was from a TBM patient co-infected with HIV.

The main difficulty with diagnosis of extrapulmonary TB in general and TBM in children in particular, is its paucibacillary nature [24, 25]. This is reflected in the low sensitivities of cultures and smears in children and could partially explain the low sensitivity found in this study [24]. It is unclear whether permeability of the blood–CSF barrier to LAM contributed to the decrease in test sensitivity from 31–64% evident in the CSF of patients with TBM [20, 21] to 4.8% observed in this study.

Mycobacterial death commences rapidly after the initiation of tuberculostatic therapy, with the majority of bacteria killed within the first few days [14]. Some patients starting treatment suffer a so-called 'paradoxical response', presumably an immunological reaction to the burst of mycobacterial antigens released when treatment starts [26, 27]. Therefore, enhanced release of LAM is expected in the first few days after treatment initiation. We tested multiple urine samples in the first 2 weeks of treatment; however, this strategy did not improve test sensitivity and no change in OD was observed over time.

We accept that our study has certain limitations. Firstly, some children were already receiving tuberculostatic therapy before hospital admission. This limited the number of urine samples taken before the start of treatment. However, positive samples were particularly expected immediately after treatment initiation; as we found no variation in OD over time, we do not believe that this influenced our results. Secondly, a case definition based on consensus [17]. was used as the gold standard for TBM diagnosis. This could have caused misclassification bias. Only four

of the patients diagnosed with TBM were classified as having Definite TBM following a positive culture and/or nucleic acid amplification test. Test sensitivity could have been underestimated as a result of the inclusion of Probable TBM cases in the TBM Group, however given the findings, subgroup analysis would not have changed outcome. Thirdly, the Clearview TB ELISA test is licensed for use only as a screening test in HIV-positive patients with suspected TB: evaluations of its use in the HIV-negative population must be considered off-label and could have contributed to the low sensitivity observed.

CONCLUSIONS

Urinary LAM detection is of little value for the diagnosis of TBM in an unselected cohort of paediatric patients. These findings are consistent with a recently published systematic review and meta-analysis by Minion *et al*. [13]. Serial measurements after treatment initiation did not improve test performance. Even though patient numbers were small, the results are so evident and in line with previous research that further testing of this particular patient group will yield no results. Urine LAM detection in TBM suspects with HIV co-infection may, however, be worth investigating further for diagnostic value. In TBM suspects without HIV co-infection, testing LAM in CSF rather than in urine, might be more informative.

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7 Improved diagnosis of childhood tuberculous meningitis using more than one nucleic acid amplification test

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ABSTRACT

Background – Early treatment is critical to reduce tuberculous meningitis (TBM)-related morbidity and mortality. Diagnosis based on cerebrospinal fluid (CSF) culture is impractical due to slow turn-around times, while microscopy has poor sensitivity. Enhanced detection methods are essential to guide early treatment initiation, especially in vulnerable young children.

Methods – We assessed the diagnostic accuracy of Genotype MTBDR*plus*® and Xpert MTB/RIF® assays on CSF collected from pediatric meningitis suspects prospectively enrolled at Tygerberg Hospital, Cape Town, South Africa. Fluorescent auramine-O microscopy, liquid culture for *Mycobacterium tuberculosis*, MTBDR*plus*® and Xpert MTB/RIF® assays were performed on all CSF samples.

Results – Of 101 meningitis suspects, 55 were diagnosed with TBM and 46 served as non-TBM controls. Using a pre-defined TBM case-definition as reference standard, sensitivities and specificities were 4% and 100% for fluorescent microscopy, 22% and 100% for culture, 33% and 98% for MTBDR*plus*®, 26% and 100% for Xpert MTB/RIF®, 22% and 100% for microscopy and/or culture combined and 49% and 98% for MTBDR*plus*® and Xpert MTB/RIF® combined. Culture, MTBDR*plus*® and Xpert MTB/RIF® combined performed best with 56% sensitivity and 98% specificity.

Conclusion – Commercial nucleic-acid amplification tests performed on CSF revealed incrementally-improved diagnostic accuracy, providing rapid microbiological confirmation but cannot serve as a rule-out test.

INTRODUCTION

In 1993, the World Health Organization declared tuberculosis (TB) a global public health emergency [1]. Although some progress has been made, patient numbers in 2012 are essentially unchanged with an estimated 8.6 million new cases and 1.3 million deaths from TB worldwide [2]. In South Africa, the TB incidence has risen to 1000 new cases/100,000 population in 2012, while large numbers of retreatment cases with a second or third episode of TB are not included in this figure [2, 3].

Tuberculous meningitis (TBM) is the most devastating manifestation of TB and early treatment initiation is critical to optimize outcomes [4]. Confirmation of TBM diagnosis is challenging in young children due to the paucibacillary nature of disease and low cerebrospinal fluid (CSF) volumes available for diagnostic analysis [5]. Currently TBM confirmation requires visualization of acid-fast bacilli and/or a positive *Mycobacterium tuberculosis* (*M. tuberculosis)* culture from CSF. Direct microscopy for acid-fast bacilli in CSF is fast but has very low sensitivity (<20%) [6] whereas mycobacterial culture may take up to 42 days and has only slightly improved sensitivity [7-9].

Several commercially available nucleic acid amplification tests (NAATs) have been developed for the rapid diagnosis of TB. The World Health Organization has endorsed the Xpert MTB/RIF® assay (Cepheid, Sunnyvale, CA, USA) for both smear microscopy-positive and -negative sputum specimens. Xpert simultaneously detects *M. tuberculosis* and susceptibility to rifampicin by amplification of the *rpo*B gene [10, 11]. It is usable for a variety of liquid clinical samples [12, 13]. However, lower sensitivities attributed to low numbers of bacilli (59-62%), were obtained for CSF specimens [14, 15].

The MTBDR*plus*® assay (Hain Lifescience GmbH, Nehren, Germany) version 1 is recommended for smear microscopy-positive specimens only [16, 17], while version 2 of the assay can also be applied to smear microscopy-negative specimens, having similar sensitivity compared to Xpert MTB/RIF® [18, 19]. The MTBDR*plus*® is a line probe assay targeting the *rpo*B, *kat*G and *inh*A genes, detecting *M. tuberculosis*, as well as rifampicin and isoniazid susceptibility. Although the MTBDR*plus*® assay version 2 has similar sensitivity and specificity to Xpert MTB/RIF® in smear microscopy-negative specimens, Xpert MTB/RIF® detects *M. tuberculosis* quicker (under 2 hours vs 5 hours) and is a closed-tube system, with easier handling and decreasing contamination rates [20].

In order to assess the utility of MTBDR*plus*® and Xpert MTB/RIF® to diagnose TBM in a clinical setting, alone and/or in combination with established diagnostic methods, we collected CSF samples from children with suspected meningitis in a setting where TBM is common.

METHODS

We conducted a prospective hospital-based study of all children clinically suspected of having meningitis.

Study Population and Setting

This study was conducted at Tygerberg Hospital, Cape Town, a major tertiary referral centre for Cape Town and surrounding areas. TBM is a common diagnosis among children diagnosed with meningitis [21]. Children were enrolled between January 2010 and March 2013. Inclusion criteria were 1) age 3 months to 13 years 2) clinical suspicion of meningitis 3) CSF sample collected for fluorescent auramine-O microscopy, *M. tuberculosis* culture, MTBDR*plus*® and Xpert MTB/RIF® assays and 4) written consent from the caregiver and assent if the child was older than 7 years and competent to do so. The study was approved by the Human Research Ethics Committee of Stellenbosch University, Cape Town, Western Cape, South Africa.

Clinical procedures

All patients underwent a comprehensive clinical evaluation. Routine investigations, including full blood count, basic biochemistry, HIV-screening, tuberculin skin test (TST), microbiological analysis of sputum or gastric washing (fluorescence microscopy for acid-fast bacilli and *M. tuberculosis* culture), bacterial blood culture, chest radiography and if clinically indicated, neuroimaging. Children were categorized as TBM, and non-TBM.

Case definitions

Tuberculous meningitis (TBM)

A diagnosis of TBM was based on a uniform research case definition (Table 1) [22]. TBM was classified as 'definite' when CSF demonstrated acid-fast bacilli and/or positive *M. tuberculosis* culture in a patient with symptoms or signs suggestive of the disease. As MTBDR*plus*® and Xpert MTB/RIF® were tested, *M. tuberculosis* detected by commercial NAATs in CSF was not used as a criteria for 'definite' TBM. TBM was classified as 'probable' or 'possible' based on a scoring system [22]. All patients diagnosed with TBM were treated with a standard short-course regimen [23].

Table 1 – Diagnostic criteria in the uniform TBM research case definition [22]

	Diagnostic score
Clinical criteria (Maximum category score=6)	
Symptom duration of more than 5 days	4
Systemic symptoms suggestive of TB (1 or more of): weight loss/ (poor weight gain in children), night sweats or persistent cough > 2 weeks	2
History of recent close contact with an individual with pulmonary TB or a positive TST/ IGRA in a child <10 years	2
Focal neurological deficit (excluding cranial nerve palsies)	1
Cranial nerve palsy	1
CSF criteria (Maximum category score=4)	
Clear appearance	1
Cells: 10–500 per µl	1
Lymphocytic predominance (>50%)	1
Protein concentration greater than 1 g/L	1
CSF to plasma glucose ratio of less than 50% or an absolute CSF glucose concentration less than 2.2mmol/L	1
Cerebral imaging criteria (Maximum category score=6)	
Hydrocephalus	1
Basal meningeal enhancement	2
Tuberculoma	2
Infarct	1
Pre-contrast basal hyperdensity	2
Evidence of tuberculosis elsewhere (Maximum category score=4)	
Chest X-ray suggestive of active TB (excluding miliary TB)	2
Chest X-ray suggestive of miliary TB	4
CT/ MRI/ US evidence for TB outside the CNS	2
AFB identified or *M.tuberculosis* cultured from another source i.e. lymph node, gastric washing, urine, blood culture	4
Exclusion of alternative diagnoses- An alternative diagnosis must be confirmed microbiologically, serologically or histopathologically	
Definite TBM = AFB seen on CSF microscopy, positive CSF *M.tuberculosis* culture, or positive CSF *M.tuberculosis* commercial NAAT in the setting of symptoms/signs suggestive of meningitis; or AFB seen in the context of histological changes consistent with TB brain or spinal cord together with suggestive symptoms/signs and CSF changes, or visible meningitis (on autopsy).	
Probable TBM = total score of ≥12 when neuroimaging available	
= total score of ≥10 when neuroimaging unavailable	
Possible TBM = total score of 6-11 when neuroimaging available	
= total score of 6-9 when neuroimaging unavailable	

TBM- tuberculous meningitis, TB- tuberculosis, TST- tuberculin skin test, IGRA- interferon gamma-release assay, CSF- cerebrospinal fluid, CT- computed tomography, MRI- magnetic resonance imaging, US- ultrasound, AFB- acid-fast bacilli, NAAT- nucleic acid amplification test

Non-TBM

This included viral, fungal or bacterial meningitis (other than TBM) and cases without meningitis (normal CSF and/or confirmation of an alternative diagnosis). Viral meningitis was confirmed when a viral pathogen was identified in the CSF by polymerase chain reaction (PCR). Viral meningitis was considered probable with clinical evidence of acute meningitis and absence of any micro-organism on Gram stain of CSF and negative routine bacterial culture of CSF if antibiotics were not administered prior to the first lumbar puncture [24]. Bacterial or fungal meningitis was determined by the identification of a bacterial pathogen in the CSF using microscopy, culture or antigen detection methods. Probable bacterial meningitis was defined as clinical evidence of meningitis in addition to a suggestive CSF examination [25].

CSF collection and testing

CSF was obtained by lumbar puncture from all children included and the following investigations performed: appearance and color determination, differential cell count determination by standard methods, protein, glucose and chloride determination by standard methods, centrifugation with Gram stain and India ink examination on the deposit and culture of the centrifuged deposit on blood agar plates for pyogenic bacteria. When viral meningitis was suspected, PCR for cytomegalovirus, Epstein-Barr virus, enteroviruses, human herpesvirus-6, herpes simplex 1 & 2 and varicella zoster, was performed on CSF. Fluorescence microscopy was conducted using standardized auramine-O staining methods [26].

M. tuberculosis culture

A volume of 0.5 ml of CSF was directly inoculated into a Mycobateria Growth Indicator Tube (MGIT; Becton Dickinson, Sparks, MD, USA) supplemented with 0.8 ml OADC (oleic acid, albumin, dextrose, catalase) containing PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, azlocillin). The MGIT was placed into a BACTEC MGIT 960 instrument, incubated at 37°C. Flagged cultures were removed and the presence of acid-fast bacilli verified by Ziehl-Neelsen staining and microscopy. Bacterial contamination was excluded by placing one drop of culture on a blood agar plate with no growth after 48 hours incubation at 37°C. Specimens were determined negative if not flagged after 42 days of incubation.

GenoType MTBDRplus®

CSF samples were processed by the National Health Laboratory Service (NHLS) TB laboratory at Tygerberg Hospital. Samples were mixed by pipetting. The Genotype MTBDR*plus®* assays were used according to the manufacturer's instructions. The CSF volume analyzed was 0.5ml, with a 160 colony forming unit (CFU)/ml limit of

detection. Quality control included a negative and positive control. Improvements in the DNA extraction from sonication and heat (version 1) to a chemical method (version 2), enabled its usage on smear microscopy-positive and -negative samples; the laboratory adopted version 2 in July 2012.

Xpert MTB/RIF®

An aliquot of 1 ml specimen was mixed with 2 ml of Xpert Sample Reagent (Cepheid), inverted 10 times, and incubated at room temperature. The inversion was repeated after 8 minutes and the incubation continued until a total duration of 15 minutes. After this, the mixture was completely transferred into an Xpert MTB/RIF cartridge, which was loaded into the GeneXpert instrument. All further processing, measuring and analysis steps happened automatically (GeneXpert Dx 4.0, Cepheid). Bacterial load was semi-quantitatively reported as very low, low, medium or high positive, with the presence or absence of resistance against rifampicin indicated separately [27]. The limit of detection is 100 CFU/ml [28]. Invalid results were repeated or excluded.

Statistical analyses

The study reporting conforms to the STARD guidelines for diagnostic accuracy reporting (www.stard-statement.org). Data analysis was performed using Statistical Package for the Social Sciences version 20 (SPSS Inc, Chicago, IL, USA). Frequencies were obtained for categorical clinical variables. Median and interquartile range was determined for continuous variables. Sensitivity, specificity, positive predictive value, negative predictive value and diagnostic odds ratio (DOR) was calculated comparing non-TBM to TBM. Categorical variables were compared using Fisher's exact test and continuous variables were compared using the Mann-Whitney U test. A p-value <0.05 was considered statistically significant.

RESULTS

In total 101 children with suspected meningitis met the inclusion criteria; 55 TBM and 46 "non-TBM". Of the TBM group, 13 patients had 'definite' TBM, 32 patients had 'probable' TBM and 10 patients had 'possible' TBM [22]. Among "non-TBM" patients, 30 did not have meningitis. Non-TBM patients with meningitis included three cases of bacterial meningitis (1 with pneumococcal meningitis) and 13 cases of viral meningitis. Confirmed viral meningitis cases included human enterovirus (5), Epstein-Barr virus (2) and Herpes simplex type-2 virus (1). CSF volume was recorded in 45 patients (36 TBM and 9 non-TBM) with a mean of 2.19 ml (95% confidence in-

terval 1.83-2.55ml). The odds ratio for CSF volume vs positive Genotype MTBDR*plus*® assay was 2.28 (95% confidence interval 1.20-4.36 p=0.012). There was no correlation between CSF volume and positive fluorescent microscopy, culture or Xpert MTB/RIF® assay. Clinical characteristics are summarized in Table 2.

Table 2 – Clinical and investigation findings of children enrolled with suspected meningitis

Clinical characteristics	**TBM**	**Non-TBM**	**p-value**
Age in months- median (IQR)	36.0 (21.0-54.0)	34.0 (17.0-56.3)	0.800
Male gender (n/N/%)	26/55 (47)	30/46 (65)	0.107
Positive HIV (n/N/%)	6/55 (11)	2/45 (4)	0.289
Positive TB contact* (n/N/%)	26/55 (47)	12/46 (26)	0.039
Poor weight gain** (n/N/%)	21/55 (38)	4/46 (9)	0.001
Hemiplegia (n/N/%)	17/55 (31)	2/46 (4)	0.001
Positive gastric washing culture (n/N/%)	25/39 (64)	11/33 (33)	0.017
Clear CSF macroscopic appearance (n/N/%)	51/55 (93)	45/45 (100)	0.125
CSF lymphocytes (cells/uL) - median (IQR)	54.0 (17.8-170.0)	1.0 (0.0-49.8)	0.515
CSF protein (g/L) - median (IQR)	1.49 (0.78-2.00)	0.25 (0.18-0.38)	0.000
CSF glucose (mmol/L) - median (IQR)	2.40 (1.10-3.40)	3.70 (3.00-4.20)	0.000
Positive AFB on CSF microscopy (n/N,%)	2/55 (4)	0/46 (0)	0.125
Positive CSF culture (n/N,%)	12/55 (22)	0/46 (0)	0.000
Positive Genotype MTBDRplus version 1 (n/N,%)	9/38 (24)	0/27 (0)	0.008
Positive Genotype MTBDRplus version 2 (n/N,%)	9/16 (56)	1/20 (5)	0.002
Positive Xpert (n/N,%)	14/55 (26)	0/46 (0)	0.000
CXR- suggestive PTB (n/N,%)	26/55 (47)	7/46 (15)	0.001
CT brain- suggestive TBM (n/N,%)	43/55 (78)	3/28 (11)	0.000

IQR= interquartile range, HIV= Human Immunodeficiency Virus, TB= tuberculosis, GCS= Glasgow coma score, CSF= cerebrospinal fluid, AFB= acid-fast bacilli, CXR= Chest X-ray, PTB= pulmonary tuberculosis, CT= computed tomography, TBM- tuberculous meningitis

*TB contact is defined as a history of recent close contact with a person with infectious tuberculosis within the past 1 year

**Poor weight gain is defined as weight loss, or slower weight gain compared to age and gender-matched controls on the WHO weight for age charts

Human immunodeficiency virus (HIV) co-infection was identified in 8 patients; 6 had neuroimaging suggestive of TBM. Of these, 5 had a positive TB contact within the last 12 months, 3 had a chest radiograph suggestive of pulmonary TB, 3 had bacteriologically-confirmed TBM and 1 had bacteriological confirmation of extraneural TB. Of the 2 non-TBM patients with HIV, 1 had confirmed pneumococcal meningitis and 1 patient did not have meningitis.

Table 3 – Sensitivity, specificity, predictive values and diagnostic odd ratios against a clinical TBM reference standard***

	TBM (n)	Non-TBM (n)	Sensitivity (95% CI)	Specificity (95% CI)	PPV	NPV	Diagnostic odds ratio (95% CI)
Total no of subjects	55	46					
Fluorescence microscopy	2	0	0.04 (0.00-0.13)	1.00 (0.92-1.00)	1.00	0.46	4.35 (0.20-92.84)
MGIT	12	0	0.22 (0.12-0.35)	1.00 (0.92-1.00)	1.00	0.52	26.72 (1.54-465.15)
Fluorescence microscopy/MGIT	12	0	0.22 (0.12-0.35)	1.00 (0.92-1.00)	1.00	0.52	26.72 (1.54-465.15)
MTBDR*plus*	18	1	0.33 (0.21-0.47)	0.98 (0.89-1.00)	0.95	0.55	21.89 (2.79-171.78)
Xpert	14	0	0.26 (0.15-0.39)	1.00 (0.92-1.00)	1.00	0.53	32.49 (1.88-561.81)
MGIT/ MTBDR*plus*	25	1	0.46 (0.32-0.59)	0.98 (0.89-1.00)	0.96	0.60	37.50 (4.82-291.73)
MGIT/Xpert	21	0	0.38 (0.25-0.52)	1.00 (0.92-1.00)	1.00	0.58	57.96 (3.39-990.22)
MTBDR*plus*/Xpert combined	27	1	0.49 (0.35-0.63)	0.98 (0.89-1.00)	0.96	0.62	43.39 (5.58-337.39)
MTBDR*plus*/Xpert/MGIT combined	31	1	0.56 (0.42-0.70)	0.98 (0.89-1.00)	0.97	0.65	58.13 (7.47-452.43)

TBM= tuberculous meningitis, MGIT= Mycobateria Growth Indicator Tube, PPV= positive predictive value, NPV= negative predictive value, CI= confidence interval, *** Uniform research case definition of Marais et al [22].

Table 4 – Sensitivity, specificity, predictive values and diagnostic odd ratios against A) a bacteriologically-confirmed (definite) TBM reference standard B) a definite and 'probable' TBM reference standard

	TBM (n)	Non-TBM (n)	Sensitivity (95% CI)	Specificity (95% CI)	PPV	NPV	Diagnostic odds ratio (95% CI)
A							
Total no of subjects	13	46					
MTBDR*plus*	12	1	0.92 (0.64-1.00)	0.98 (0.89-1.00)	0.92	0.98	540.00 (31.42-9279.80)
Xpert	5	0	0.39 (0.14-0.68)	1.00 (0.92-1.00)	1.00	0.85	60.18 (3.04-1191.80)
MTBDR*plus* /Xpert combined	12	1	0.92 (0.64-1.00)	0.98 (0.89-1.00)	0.92	0.98	540.00 (31.42-9279.80)
B							
Total no of subjects	45	46					
MTBDR*plus*	14	1	0.31 (0.18-0.47)	0.98 (0.89-1.00)	0.93	0.59	20.32 (2.54-162.62)
Xpert	14	0	0.31 (0.18-0.47)	1.00 (0.92-1.00)	1.00	0.60	42.81 (2.46-743.98)
MTBDR*plus* /Xpert combined	23	1	0.51 (0.35-0.66)	0.98 (0.89-1.00)	0.96	0.67	47.05 (5.96-371.35)

TBM= tuberculous meningitis, MGIT= Mycobateria Growth Indicator Tube, PPV= positive predictive value, NPV= negative predictive value, CI= confidence interval

The diagnostic accuracy of the CSF tests against a TBM case definition and culture-confirmed TBM is reflected in Table 3 and 4, respectively. When using a TBM case definition as the reference standard, both NAATs performed better than liquid culture and demonstrated some incremental value, although sensitivity remained sub-optimal. When using 'definite' TBM as the reference standard, the MTBDR*plus*® assay performed with 92% sensitivity and 98% specificity and Xpert MTB/RIF® assay performed with 39% sensitivity and 100% specificity in a small (n=13) group of children.

One "non-TBM" case tested positive with the Genotype MTBDR*plus*® assay version 2, but was negative on microscopy, culture and Xpert. The patient had a CSF picture suggestive of viral meningitis, and CSF PCR confirmation of Epstein-Barr virus, as well as normal brain computed tomography (CT). The patient improved clinically without any TB treatment and likely represented a false-positive test. Laboratory cross-contamination could not be ruled out with certainty, but no other cases of potential cross-contamination were detected.

An *inh*A mutation was detected in one patient, using the Genotype MTBDR*plus*® assay version 2, which is usually associated with low-level isoniazid resistance. This specific patient's treatment regimen included high dose isoniazid (20mg/kg), along with high dose rifampicin (20mg/kg), ethionamide (20mg/kg) and pyrazinamide (40mg/kg). As this patient defaulted treatment for 1 month, the total treatment period was 12 months. The patient was clinically followed up at 1-monthly intervals, with consistent weight-gain throughout. After 12 months of therapy the patient was considered cured, and discharged.

DISCUSSION

The main finding of this study is the incremental increase in diagnostic accuracy that can be achieved with commercial NAATs performed on CSF. Although both NAATs were superior to liquid culture, sensitivity remained low compared to a rigorous predefined clinical case definition. Combining any positive NAAT provided a sensitivity of 49%, which is insufficient to serve as a rule-out test and provides limited clinical guidance. However, a positive test provides useful microbiological confirmation with rapid turn-around times. When compared to culture-confirmed TBM, both NAATs performed with better sensitivities (especially MTBDR*plus*® assay with sensitivity 92%), however patient numbers in this group were small.

A recent meta-analysis of the accuracy of commercial NAATs for the diagnosis of TBM revealed a pooled sensitivity and specificity of 64% and 98%. These studies used culture-confirmed TBM as the reference standard [29], a group where higher sensitivities would have been expected. A uniform research case definition proposed for adults and children state that a TBM diagnosis can be regarded as "definite" when *M. tuberculosis* is cultured from CSF and/or a commercial NAAT is positive for *M. tuberculosis* [22]. Our findings support this position, since only a single NAAT test was considered to be a false positive test; likely the result of laboratory contamination. This emphasizes the importance of ensuring optimal laboratory infection and contamination control standards.

The relatively poor correlation between NAATs and liquid culture may reflect the fact that NAATs detect DNA from viable and non-viable bacteria. Although every attempt was made to collect the CSF sample prior to the initiation of empiric therapy, some children were referred from outside centers and received initial treatment prior to CSF collection. This could explain the relatively low culture yields achieved, but it cannot explain why only a minority of cases with positive NAAT were both MTBDR*plus*® and Xpert MTB/RIF® positive. NAAT discrepancy may be due to random sampling variation in a pauci-bacillary CSF specimen. It has been suggested that at least 6 ml of CSF should be collected and concentrated to improve the diagnostic yield [30]. From our paediatric population we could only obtain a mean of 2.19 ml of CSF, and splitting these low volumes for four different tests could have resulted in false negative tests in instances where the bacterial load was below detection threshold. However, low CSF volumes are an unfortunate clinical reality in young children and in clinical practice all these tests will not have to be performed in parallel. Even with the low CSF volume obtained, the yield for the MTBDR*plus*® assay increased significantly with increased CSF volume.

The sensitivity of fluorescence microscopy (4%) was lower than that reported in the literature (10-20%) [6] and that of MGIT liquid culture was comparable (26% vs 22%) [31]. There are no studies describing the use of the MTBDR*plus*® assay in CSF samples of either adults or children. The sensitivity of 33% (98% specificity) against a TBM case definition and sensitivity of 98% (98% specificity) against microbiologically-confirmed TBM, is encouraging and compares favorably with the performance on smear microscopy-negative sputa (19%) [32]. Xpert was 26% sensitive (100% specificity) against a TBM case definition and 39% sensitive (100% specificity) against microbiologically-confirmed TBM, but the use of this assay on CSF is not yet that well described. A pooled sensitivity of 70%; specificity of 97% for Xpert MTB/RIF® compared to liquid culture as a reference standard, was obtained in five studies in

a recent meta-analysis [13, 14, 30, 33-35]. Concentration steps could have helped to reach the Xpert MTB/RIF® assay's detection threshold of approximately 100 bacteria/ml [13].

Our clinical practice is to start anti-tuberculosis treatment on clinical suspicion prior to bacteriological confirmation. In settings with low TB incidence where experience with TBM is limited, treatment can be delayed with potentially dire consequences. In such settings NAATs offer improved CSF sensitivity, with good specificity, and a potential for same day diagnosis. The cost of the NAAT assays needs to be put into perspective to potential cost-savings by shorter hospital stay and better outcomes due to earlier initiation of treatment.

CONCLUSIONS

Commercial NAATs performed on CSF revealed incremental improvement in sensitivity, with specificity maintained. The best sensitivity was obtained with the combination of liquid culture and both NAATs, but there is not a massive gain when compared to both NAATs only. However, NAATs alone or in combination, cannot serve as a rule out test but can provide rapid microbiological confirmation. Cost-analysis needs to be performed comparing the expense of NAATs to the potential cost saving of early initiation of treatment and shorter hospital stay.

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8 The impact of drug resistance on clinical outcome in children with tuberculous meningitis

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ABSTRACT

Background – Tuberculous meningitis (TBM) is associated with delayed diagnosis and poor outcome in children. This study investigated the impact of drug resistance on clinical outcome in children with TBM.

Methods – All children (0-13 years) were included if admitted to Tygerberg Children's Hospital, Cape Town, South Africa from January 2003 to April 2009 with a diagnosis of either confirmed TBM, or probable TBM with mycobacterial isolation from a site other than cerebrospinal fluid. Mycobacterial samples underwent drug susceptibility testing to rifampin and isoniazid. Children were treated with isoniazid, rifampin, pyrazinamide and ethionamide according to local guidelines.

Results – 123 children were included; 13% (16 of 123) had any form of drug resistance, 4% (5 of 123) had multidrug-resistant (MDR)-TB. Time from start of symptoms to appropriate treatment was longer in children with any drug resistance (median: 31 days vs. 9 days; p=0.001). In multivariable analysis young age (p=0.013) and MDR-TB (adjusted OR: 12.4 [95%CI: 1.17-132.3]; p=0.037) remained risk factors for unfavourable outcome, and MDR-TB remained a risk for death (adjusted OR: 63.9 [95%CI: 4.84-843.2]; p=0.002). We did not detect any difference in outcome between those with isolates resistant to only isoniazid and those with fully susceptible stains (OR: 0.22 [CI: 0.03-1.87]; p=0.17).

Conclusion – MDR-TBM in children has poor clinical outcome and is associated with death. We did not find any difference in the outcomes between children with isoniazid mono-resistant TBM and those with drug-susceptible TBM. One explanation may be the local treatment regimen. Further investigation of this regimen is indicated.

INTRODUCTION

Tuberculous meningitis (TBM) is a severe form of tuberculosis (TB) and frequently occurs in early childhood [1]. Haematogenous spread of bacilli from a primary pulmonary focus leads to the development of a Rich focus in the brain. Rupture of this caseous granuloma into the subarachnoid space causes the clinical features of TBM [2, 3]. This usually starts insidiously with a prodromal period of non-specific symptoms but as the disease progresses, neck stiffness, loss of consciousness, motor paresis and convulsions invariably follow. The diagnosis is often delayed and only considered once irreversible neurological damage has already occurred [1, 4]. Untreated, the condition is almost universally fatal with a median time to death of 19.5 days [5]. Even for those treated, TBM is associated with high rates of mortality and morbidity; about 80% of children with advanced disease at diagnosis (TBM stage II and TBM stage III) will suffer severe neurological sequelae [1, 4]. TBM is the commonest cause of bacterial meningitis in the Western Cape Province (WCP) of South Africa [6].

The World Health Organization (WHO) estimated that there were 440,000 new cases of multidrug-resistant (MDR)-TB globally during 2008 [7]. MDR-TB is caused by *Mycobacterium tuberculosis* (*M. tuberculosis*) resistant to both isoniazid and rifampin. Extensively drug-resistant (XDR)-TB is additionally resistant to a fluoroquinolone and an injectable second-line anti-TB medication. As TB in children is usually paucibacillary, microbiological diagnosis only occurs in 20-40% of cases with evidence of disease [8]. As drug susceptibility testing (DST) requires a microbiological diagnosis, the diagnosis of MDR-TB in children is often made presumptively. This is based on signs, symptoms and radiology suggestive of TB in the context of either an MDR-TB source case or treatment failing in a child being treated with a first-line regimen. As an MDR-TB source case is not always identified, most children with MDR-TB are initially treated with a first-line regimen until their culture and DST results are available, an MDR-TB source case is identified or treatment is found to be failing. The initiation of appropriate treatment with second-line drugs is therefore often delayed in children with MDR-TB [9, 10].

MDR-TBM has very poor outcome [11 – 14] but there are little data regarding children. The relationship between the *M. tuberculosis* strain and clinical phenotype has been explored in both adults and children with TBM [15 – 17] with conflicting results. The relationship between strain type and drug resistance pattern is complex but a strong association exists between drug resistance and the Beijing genotype [18 – 20]. The aim of this study is to analyse whether a relationship exists between the drug susceptibility pattern of the infecting *M. tuberculosis* organism and the clinical outcome of TBM in children and to determine if this relationship is influenced by the genotype of the strain.

PATIENTS AND METHODS

Setting

Tygerberg Children's Hospital (TCH), in the WCP, South Africa, provides specialized care to half of the province's 1.2 million children. The WCP had a TB notification rate of 978 per 100,000 in 2009 [21] and amongst all children with routinely diagnosed culture-confirmed TB at the TCH during 2005-2007, 6.7% were identified as MDR [22].

Study population and TBM definition

All children admitted to TCH from January 2003 until April 2009, aged 0-13 years, were included if they had either a diagnosis of confirmed TBM (*M. tuberculosis* isolated from the cerebrospinal fluid [CSF]), or of probable TBM with a positive culture of *M. tuberculosis* from a source other than the CSF. Probable TBM was defined as a clinical diagnosis of meningitis, supported by the presence of characteristic CSF findings (pleiocytosis, elevated protein level and reduced glucose level). In addition, two or more of the following criteria were required: recent weight loss, a positive tuberculin skin test (TST), a chest radiograph (CR) compatible with TB, a cranial computerized tomography (CT) scan compatible with TBM or finally, household contact with sputum smear-positive pulmonary TB [4].

Clinical care

In the WCP, HIV-uninfected children with TBM are treated with isoniazid (20mg/kg, maximum 400mg daily), rifampin (20mg/kg, maximum 600mg daily), pyrazinamide (40mg/kg, maximum 2g daily) and ethionamide (20mg/kg, maximum 1g daily) for six months with HIV-infected children treated for nine months. If the child's isolate of *M. tuberculosis*, or that of the source case, is resistant to any of the drugs used in the local TBM treatment regimen, or if the child deteriorates clinically on this regimen, alternative anti-TB treatment is considered. Treatment is tailored to the DST of the child or source case's isolate. If diagnosed in the context of a failing regimen, treatment is directed at the DST of locally prevailing strains. Treatment for isoniazid mono-resistant (HMR)-TB involves the addition of a fluoroquinolone and terizidone with treatment for nine months irrespective of HIV status. Treatment of MDR- and rifampin mono-resistant (RMR)-TB includes any first-line drugs to which the organisms are susceptible, a second-line injectable medication, a fluoroquinolone, and further drugs (from WHO classes four and five) to make up at least four effective drugs with good CSF penetration [23 – 25]. Treatment for MDR (and RMR)-TB, for both HIV-infected and -uninfected children, typically consists of six months of intensive phase therapy including an injectable medication followed by a further twelve months of oral therapy.

Children are treated as inpatients at TCH or Brooklyn Chest Hospital (BCH; a TB referral hospital) unless social circumstances are assessed and deemed satisfactory for a home-based care programme. MDR-TBM patients are treated in hospital for at least the intensive phase. All children are treated with steroids. An air encephalogram is performed if there is evidence of hydrocephalus on CT scan; if non-communicating, a ventriculoperitoneal shunt is inserted. HIV testing is performed following informed consent from the parent or legal guardian using ELISA if older than 18 months or DNA PCR if younger or breast-fed. Antiretroviral therapy (ART) is initiated as soon after HIV diagnosis as is possible. TST is performed by injecting two tuberculin units intradermally (purified protein derivative RT23, Statens Serum Institute) with results read at 48-72 hours. A transverse diameter of ≥10mm is considered positive in HIV-uninfected and ≥5mm in HIV-infected children.

Data collection

Every child with culture-confirmed TB at TCH is recorded prospectively in a clinical database with DST to rifampin and isoniazid routinely performed on a single sample from all children. A list of children with a diagnosis of TBM was extracted from the database. Case notes were retrieved for these children from TCH and BCH to confirm inclusion criteria and extract clinical details. Patients were included if there was complete documentation of presentation, clinical course and outcome. Development Quotient (DQ) was measured at the end of TB treatment using the Bayley test, Griffiths test or Junior South African Individual Scale, dependant on age. Visual testing was performed clinically. In the majority, formal assessments had been performed by a developmental paediatrician but for some children, an outcome was assigned by the study team based on clinical examinations that had been undertaken by paediatric neurologists, general paediatricians, paediatric registrars or medical officers. For those with complete clinical details, isolates underwent spoligotype analysis.

Mycobacterial culture and DST

Respiratory samples were inoculated into Middlebrook 7H9 broth-based Mycobacterial Growth Indicator Tubes (MGIT; Becton Dickinson, Sparks, MD, USA) following a standard protocol for decontamination, while samples from sterile sites, including CSF, were inoculated directly. *M. tuberculosis* complex isolates were confirmed as *M. tuberculosis* through PCR [26]. From January 2003 until August 2008 conventional, phenotypic DST was by the indirect proportion method [22]. From August 2008 genotypic DST was performed using the GenoType® MTBDRplus (Hain Life Science, Nehren, Germany) line probe assay, carried out according to the manufacturer's instructions [27].

Spoligotyping

Genotype determination was performed using standardized spoligotyping methodology [28]. Isolates were assigned to specific genotype families according to their spoligotype signature which included the internationally recognized families of Beijing, LAM (Latin American and Mediterranean family), Haarlem, CAS (Central Asian lineage), a group of ill-defined strains of the T family, LCC (Low Copy Number Clade) and S family [29, 30]. It was not possible to classify some of the remaining strains.

Data classification

The time from first reported symptoms to initiation of anti-TB therapy was recorded. In cases of drug-resistant TBM, the time from the first reported symptoms to appropriate second-line therapy was also determined. TBM stage was classified as TBM stage I (Glasgow Coma Scale [GCS] 15 with no focal neurological signs), TBM stage II (GCS 11-14 or GCS 15 with focal neurology) or TBM stage III (GCS <11) [4]. GCS (or modified pediatric GCS) was assessed and recorded at the time of presentation by the attending doctor. HIV immunological staging was based on WHO criteria [31]. Although the identified strains were recorded, strains were classified as simply Beijing or non-Beijing for analysis. DST was recorded as drug-susceptible (DS), HMR, RMR and MDR. Motor function at the end of therapy was classified as normal, hemiparesis or quadriparesis, cognitive function as normal (DQ >80), mild handicap (DQ: 50-80) or severe handicap (DQ<50) and vision as normal, impaired vision or blind. For analysis, we looked at two dichotomous outcome measures: mortality (alive or dead) and clinical outcome (favourable or unfavourable). A child was classed as having an unfavourable outcome if they died or were left with quadriparesis, severe cognitive handicap or blindness.

Statistical analysis

Data were analysed using STATA version 11 with missing data excluded from analysis. Continuous variables were used for age, time to initiation of appropriate therapy and CSF parameters; all other data were categorical. Associations were assessed using the Fisher's exact test when comparing categorical data with the effect estimated (odds ratios; OR) and 95% confidence intervals (CI) calculated. The Mann Whitney test was used to assess the effect of age, treatment delay and CSF measurements, given the non-normal distribution of the data with median and inter-quartile range (IQR).

Risk factors for the two outcomes (unfavourable clinical outcome and death) were assessed in univariate analysis. Multivariable models were used to analyse the relationship between risk factors and outcome if either the univariate relationships showed significance ($p<0.05$) or where variables were thought to be clinically relevant. Standard tests for co-linearity were used.

Ethics

Ethical approval was obtained from the Stellenbosch University Human Research Ethics Committee and the London School of Hygiene and Tropical Medicine.

RESULTS

Patient characteristics

One hundred and forty-two children were identified from the database of children with culture-confirmed TB. On review of the clinical records five did not meet the inclusion criteria. Of the remaining 137 cases, comprehensive clinical details could be found on 123 (see Figure 1). The baseline clinical characteristics of these patients are demonstrated in Table 1 with the initial investigations, clinical course and outcome in Table 2. For 104 of these patients samples were located and spoligotyping successfully performed. Ninety-eight (79.7%) of the 123 children included in the analysis were tested for HIV, and of these 20 (20.4%) were HIV-infected.

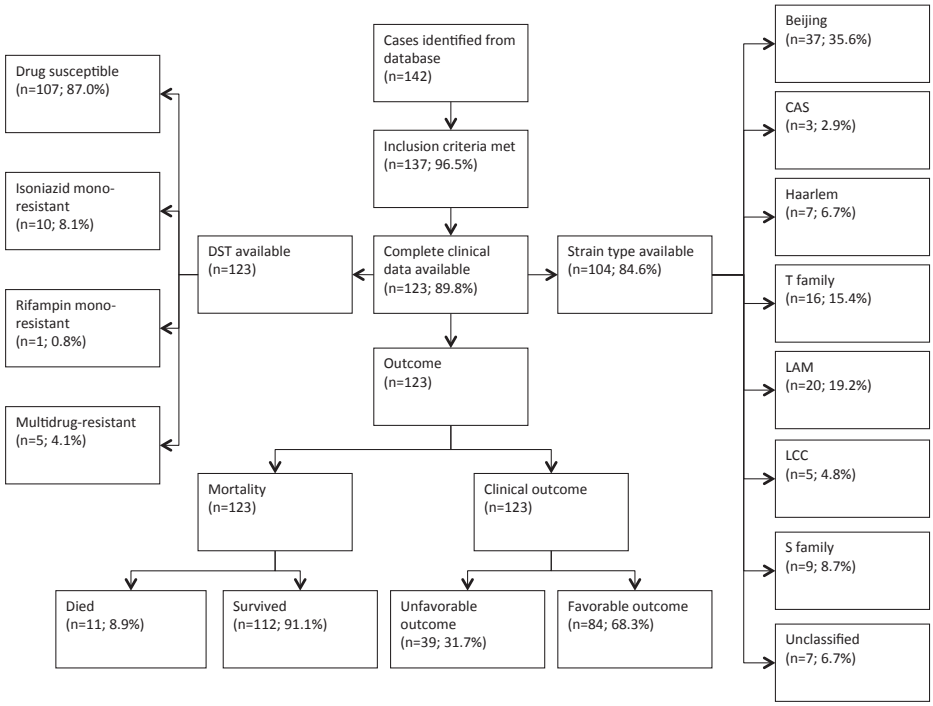

Figure 1: Patient identification, inclusion, mycobacterial characteristics and outcome
DST – drug susceptibility test; CAS – Central Asian Strain; LAM – Latin American Mediterranean; LCC – Low-copy-number Clade

Table 1 – Presenting clinical characteristics (n=123 unless otherwise stated)

	DS (107)	HMR (10)	RMR (1)	MDR (5)	Total (123)
Age (median & IQR in months)	28 (12-56)	25 (21-38)	14	26 (26-50)	27 (13-55)
Male gender (%)	53 (49.5)	5 (50)	0	1 (20)	59 (48.0)
HIV-infected (n=98; %)	16 (19.3)	1 (11.1)	0	3 (60)	20 (20.4)
Evidence of BCG (n=111; %)	83 (85.6)	7 (87.5)	1	4 (80)	95 (85.6)
TB contact history (n=116; %)	61 (60.4)	7 (77.8)	1	4 (80)	73 (62.9)
Preventive/previous treatment (n=122; %)	11 (10.4)	2 (20)	1**	4 (80)*	18 (14.8)
TST positive (n=108; %)	64 (68.8)	7 (77.8)	0	3 (60)	74 (68.5)
Time from start of symptoms to treatment initiation (median & IQR in days)	9 (5-21)	16 (14-30)	3	6 (2-19)	9 (5-21)
Time from start of symptoms to appropriate treatment initiation (median & IQR in days)	9 (5-21)	31 (14-53)	82	19 (6-51)	11 (5-22)
Presenting symptoms (more than one in most cases; %)					
Decreased consciousness	57 (53.3)	5 (50)	0	5 (100)	67 (54.5)
Headache	26 (24.3)	4 (40)	0	0	30 (24.4)
GI disturbance	16 (15.0)	2 (20)	0	1 (20)	19 (15.5)
Poor feeding	17 (15.9)	1 (10)	0	0	18 (14.6)
Seizures	47 (43.9)	5 (50)	0	2 (40)	54 (43.9)
Vomiting	50 (46.7)	3 (30)	0	2 (40)	55 (44.7)
Cough	40 (37.7)	4 (40)	1	3 (60)	48 (39.3)
Weight loss	93 (86.9)	8 (80)	1	5 (100)	107 (87.0)
Fever	72 (67.3)	8 (80)	1	4 (80)	85 (69.1)
Irritability	9 (8.4)	0	0	0	9 (7.3)
Lethargy	30 (28.0)	1 (10)	1	1 (20)	32 (26.0)
Neck stiffness	23 (21.5)	1 (10)	1	1 (20)	25 (20.3)
TBM stage (%) I	22 (20.1)	3 (30)	1		26 (21.1)
II	44 (41.1)	5 (50)	0	1 (20)	50 (40.7)
III	41 (38.3)	2 (20)	0	4 (80)	47 (38.2)
GCS (median & IQR)	12 (9-15)	14 (11-15)	15	6 (5-11)	12 (9-15)
Cranial nerve abnormalities noted at presentation (%)	52 (48.6)	8 (80)	1	3 (60)	57 (46.3)
Motor abnormalities noted at presentation (%)	72 (67.3)	2 (20)	1	0	85 (69.1)

TB = tuberculosis; TBM = TB meningitis TST = tuberculin skin test; DS = drug-susceptible; HMR = isoniazid monoresistant; RMR = rifampin monoresistant; MDR = multidrug resistant; IQR = inter-quartile range; GCS = Glasgow coma scale

* One child developed TBM whilst on first-line treatment for pulmonary TB. One child was given isoniazid prophylaxis, one child developed TBM whilst on treatment for confirmed MDR-TB (suspicion of XDR-TB). One child had MDR-TB prophylaxis (isoniazid, ethambutol and ofloxacin) from birth but was then re-exposed over a year later and developed MDR-TBM. The final child received no preventive treatment.

** This child was prescribed isoniazid and rifampin prophylaxis at birth, but it was not given. The child presented almost a year later with Stage I TBM. Nearly three months later resistance testing showed RMR, and although clinically well, treatment was changed to MDR-TB treatment. However, she died two months later after sudden deterioration.

Table 2 – Investigations at diagnosis, clinical course and outcome (n=123 unless otherwise stated)

	DS (107)	HMR (10)	RMR (1)	MDR (5)	Total (123)
Diagnosis (%)					
Confirmed TBM	23 (21.5)	2 (20)	1	4 (80)	30 (24.4)
Probable TBM	84 (78.5)	8 (80)	0	1 (20)	93 (75.6)
Strain (n=104; %)					
Beijing	30 (33.0)	6 (60)	0	1 (33.3)	37 (35.6)
LAM	18 (19.8)	2 (20)	0	0	20 (19.2)
Haarlem	5 (5.5)	1 (1)	1	0	7 (6.7)
CAS	3 (3.3)	0	0	0	3 (2.9)
Ill-defined T family	16 (17.6)	0	0	0	16 (15.4)
LCC	5 (5.5)	0	0	0	5 (4.8)
S family	7 (7.7)	0	0	2 (66.7)	9 (8.7)
Undefined	7 (7.7)	0	0	0	7 (6.7)
CSF Lymphocytes (n=116; median & IQR)	57 (24-138)	28 (5-130)	73	75 (35-105)	57 (22-132)
CSF Protein (n=108; median & IQR)	1.3 (0.9-2.1)	1.3 (0.4-2.2)	1.2	8.4 (1.3-15.5)	1.4 (0.9-2.1)
Air encephalogram (n=61; %)					
Non-communicating hydrocephalus	19 (34.6)	1 (25)	-	1 (50)	23 (37.7)
Communicating hydrocephalus	36 (65.5)	3 (75)	-	1 (50)	38 (62.3)
Ventriculoperitoneal shunt inserted (%)	25 (23.4)	2 (20)	0	2 (40)	29 (23.6)
Admitted to intensive care (%)	13 (12.2)	0 (0)	0	4 (80)	17 (13.8)
Duration hospital admission (median & IQR in days)	27 (16-38)	32 (18-53)	35	36 (29-40)	28 (17-39)
Motor function amongst survivors at end of therapy (n=112; %)					
Normal	54 (53.5)	8 (80)	0	1	63 (56.3)
Hemiparesis	34 (33.7)	2 (20)	0	0	36 (32.1)
Quadriparesis	13 (12.9)	0 (0)	0	0	13 (11.6)
Cognitive function amongst survivors at end of therapy (n=112; %)					
Normal	37 (36.6)	5 (50)	0	0	42 (37.5)
Mild handicap	37 (36.6)	4 (40)	0	1	42 (37.5)
Severe handicap	27 (26.7)	1 (10)	0	0	28 (25.0)
Vision at amongst survivors at end of therapy (n=108; %)					
Normal	77 (79.4)	9 (90)	0	1	87 (80.6)
Impaired vision	15 (15.5)	1 (10)	0	0	16 (14.8)
Blind	5 (5.2)	0 (0)	0	0	5 (4.6)
Mortality (%)					
Survived	101 (94.4)	10 (100)	0	1 (20)	112 (91.1)
Died	6 (5.6)	0 (0)	1	4 (80)	11 (8.9)
Clinical outcome (%)					
Favourable	74 (69.2)	9 (90)	0	1 (20)	84 (68.3)
Unfavourable	33 (30.8)	1 (10)	1	4 (80)	39 (31.7)

TB = tuberculosis; TBM = TB meningitis TST = tuberculin skin test; DS = drug-susceptible; HMR = isoniazid monoresistant; RMR = rifampin monoresistant; MDR = multidrug resistant; IQR = inter-quartile range; GCS = Glasgow coma scale; CSF = cerebro-spinal fluid; CAS – Central Asian Strain; LAM – Latin American Mediterranean; LCC – Low-copy-number Clade

Six (30.0%) of the HIV-infected children had severe immunosuppression at the time of TBM diagnosis, and only three (15.0%) were on ART.

Drug resistance, strain type and outcome

Sixteen children (13.0%) had isolates with drug resistance, five MDR (4.1%), ten HMR (8.1%) and one RMR (0.8%). No XDR-TB cases were identified. Univariate analysis showed an association between MDR-TB and both poor clinical outcome (OR 8.97; 95%CI 0.83-4447.5; p=0.04) and death (OR 67.3, 95%CI 5.0-3343; p<0.001) as shown in Table 3. There was no association between Beijing strain and unfavourable outcome (p=0.29) or mortality (p=1.0). In addition, there was no relationship between Beijing strain and any drug resistance (p=0.21) or MDR (p=1.00). A trend towards an association existed between MDR-TB and HIV (OR 6.71, 95%CI 0.69-83.7; p=0.056), but not with TBM stage (p=0.22). Beijing strain was not associated with HIV status (p=0.78) or TBM stage (p=0.14).

Clinical factors and outcome

Children with unfavourable outcome were younger than those with favourable outcome (median age: 21 months [IQR: 7-35] vs. 30 months [IQR: 15-72]; p=0.008). They also had lower CSF lymphocyte counts (median: 35 cells/µl [IQR: 17-61] vs. 75 cells/µl [IQR: 27-159]; p=0.002). CSF lymphocyte counts were not associated with HIV infection (p=0.24) or strain type (p=0.07). TBM stage III (p<0.001), shunt insertion (p=0.002) and intensive care admission (p=0.02) were associated with unfavourable outcome and reflect disease severity (Table 3). HIV infection was associated with death (OR 6.17, 95%CI 1.15-34.1; p=0.02) and for those dying the time from first symptoms to appropriate treatment was longer (median: 22 days [IQR: 6-61] vs. 10 days [IQR: 5-21]; p=0.049). Time from start of symptoms to initiation of appropriate TB treatment was longer for those with any drug-resistance than those with drug-susceptible TBM (median: 31 days [IQR: 13-66] vs. 9 days [IQR: 5-21]; p=0.001). Time to start appropriate therapy was not influenced by the presence of a known TB source case (p=0.82), the HIV status of the child (p=0.10) or the age of the child (p=0.82).

Multivariable analysis

Following adjustment for HIV status in multivariate analysis the relationship between MDR-TB and death persisted (adjusted OR 63.9, 95%CI 4.84-843.2; p=0.0002; see Table 4)). Young age (p=0.013) and MDR-TB (adjusted OR 12.4, 95%CI 1.17-132.3; p=0.037) remained independent risk factors for unfavourable outcome. Those with HMR-TB did not have an increased risk of unfavourable outcome after adjustment for age (adjusted OR 0.22, 95%CI 0.03-1.87; p=0.17). The relationship between HIV and death

Table 3 – Univariate relationship between microbiological factors, clinical characteristics and outcome

			Unfavourable outcome			Death	
		Total N	**N**	**OR (95%CI)**	**p-value**	**N**	**OR (95%CI)**	**p-value**
DST								
	DS	107	33	1.00		6	1.00	
	HMR	10	1	0.25 (0.01-1.95)	0.28#	0	-	1.0#
	RMR	1	1	-	0.32#	1	-	0.06#
	MDR	5	4	8.97 (0.83-447.5)	0.04#	4	67.3 (5.0-3343)	<0.001#
Strain								
	Beijing	37	15	1.00		3	1.00	
	Non-Beijing	67	20	0.62 (0.25-1.58)	0.29#	6	1.11 (0.22-7.32)	1.0#
HIV status								
	Negative	78	24	1.00		4	1.00	
	Positive	20	9	1.84 (0.59-5.61)	0.29#	5	6.17 (1.15-34.1)	0.02#
Age*					0.008##			0.34##
Gender	Female	64	23	1.00		8	1.00	
	Male	59	16	0.66 (0.28-1.53)	0.34#	3	0.38 (0.061-1.68)	0.21#
BCG status								
	None	16	4	1.00		2	1.00	
	Given	95	34	1.67 (0.46-7.64)	0.57#	9	0.73 (0.13-7.69)	0.66#
TBM stage								
	I	26	4	1.00		3	1.00	
	II	50	3	0.35 (0.05-2.30)	0.22#	1	0.15 (0.003-2.12)	0.11#
	III	47	32	11.7 (3.10-53.2)	<0.001#	7	1.34 (0.27-8.78)	1.0#
Time to appropriate therapy**					0.98##			0.049##
CSF Lymphocyte count***					0.002##			0.54##
CSF Protein** **					0.88##			0.16##
Ventriculoperitoneal shunt								
	No	94	23	1.00		9	1.00	
	Yes	29	16	3.80 (1.45-9.94)	0.002#	2	0.70 (0.07-3.70)	1.0#
Admission to intensive care								
	No	106	29	1.00		6	1.00	
	Yes	17	10	3.79 (1.16-12.8)	0.02#	5	6.94 (1.41-31.6)	0.008#

OR = odds ratio, CI = confidence interval, TBM = tuberculous meningitis, CSF = cerebrospinal fluid; DST = drug susceptibility test; DS = drug-susceptible; HMR = isoniazid monoresistant; RMR = rifampin monoresistant; MDR = multidrug resistant.
Unfavourable outcome – death, quadriparesis, severe cognitive handicap or blindness
Fisher's Exact test used; ## Mann Whitney test used
* Median age in months: Favourable outcome: 30; Unfavourable outcome: 21; Survival: 28; Death: 26
** Median time in days: Favourable outcome: 9; Unfavourable outcome: 14; Survival: 10; Death: 22
*** Median count: Favourable outcome: 75; Unfavourable outcome: 35; Survival: 57; Death: 39
**** Median value: Favourable outcome: 1.32; Unfavourable outcome: 1.41; Survival: 1.32; Death: 2.0

Table 3a - Univariate relationship between microbiological factors, clinical characteristics and clinical outcome

		Outcome				
		Total N	**Favourable**	**Unfavourable**	**OR (95%CI)**	**p-value**
DST	DS	107	74	33	1.00	
	HMR	10	9	1	0.25 (0.01-1.95)	0.28#
	RMR	1	0	1	-	0.32#
	MDR	5	1	4	8.97 (0.83-447.5)	0.04#
Strain	Beijing	37	22	15	1.00	
	Non-Beijing	67	47	20	0.62 (0.25-1.58)	0.29#
HIV status	Negative	78	54	24	1.00	
	Positive	20	11	9	1.84 (0.59-5.61)	0.29#
Age*						0.008##
Gender	Female	64	41	23	1.00	
	Male	59	43	16	0.66 (0.28-1.53)	0.34#
BCG status	None	16	12	4	1.00	
	Given	95	61	34	1.67 (0.46-7.64)	0.57#
TBM stage	I	26	22	4	1.00	
	II	50	47	3	0.35 (0.05-2.30)	0.22#
	III	47	15	32	11.7 (3.10-53.2)	<0.001#
Time to appropriate therapy**						0.98##
CSF Lymphocyte count***						0.002##
CSF Protein****						0.88##
Ventriculoperitoneal shunt inserted						
	No	94	71	23	1.00	
	Yes	29	13	16	3.80 (1.45-9.94)	0.002#
Admission to intensive care						
	No	106	77	29	1.00	
	Yes	17	7	10	3.79 (1.16-12.8)	0.02#

OR = odds ratio, CI = confidence interval, TBM = tuberculous meningitis, CSF = cerebrospinal fluid; DST = drug susceptibility test; DS = drug-susceptible; HMR = isoniazid monoresistant; RMR = rifampin monoresistant; MDR = multidrug resistant.

Unfavourable outcome – death, quadriparesis, severe cognitive handicap or blindness

\# Fisher's Exact test used; ## Mann Whitney test used

* Median age in months: Favourable outcome: 30; Unfavourable outcome: 21

** Median time in days: Favourable outcome: 9; Unfavourable outcome: 14

*** Median count: Favourable outcome: 75; Unfavourable outcome: 35

**** Median value: Favourable outcome: 1.32; Unfavourable outcome: 1.41

Table 3b - Univariate relationship between microbiological factors, clinical characteristics and death

		Total	Survivors	Deaths	OR (95%CI)	p-value
		N	N	N		
DST	DS	107	101	6	1.00	
	HMR	10	10	0	-	1.0#
	RMR	1	0	1	-	0.06#
	MDR	5	1	4	67.3 (5.0-3343)	<0.001#
Strain	Beijing	37	34	3	1.00	
	Non-Beijing	67	61	6	1.11 (0.22-7.32)	1.0#
HIV status	Negative	78	74	4	1.00	
	Positive	20	15	5	6.17 (1.15-34.1)	0.02#
Age*						0.34##
Gender	Female	64	56	8	1.00	
	Male	59	56	3	0.38 (0.061-1.68)	0.21#
BCG status	None	16	14	2	1.00	
	Given	95	86	9	0.73 (0.13-7.69)	0.66#
TBM stage	I	26	23	3	1.00	
	II	50	49	1	0.15 (0.003-2.12)	0.11#
	III	47	40	7	1.34 (0.27-8.78)	1.0#
Time to appropriate therapy**						0.049##
CSF Lymphocyte count***						0.54##
CSF Protein****						0.16##
Ventriculoperitoneal shunt inserted						
	No	94	85	9	1.00	
	Yes	29	27	2	0.70 (0.07-3.70)	1.0#
Admission to intensive care						
	No	106	100	6	1.00	
	Yes	17	12	5	6.94 (1.41-31.6)	0.008#

OR = odds ratio, CI = confidence interval, TBM = tuberculous meningitis, CSF = cerebrospinal fluid; DST = drug susceptibility test; DS = drug-susceptible; HMR = isoniazid monoresistant; RMR = rifampin monoresistant; MDR = multidrug resistant.

\# Fisher's Exact test used; ## Mann Whitney test used

* Median age in months: Survival: 28; Death: 26

** Median time in days: Survival: 10; Death: 22

*** Median count: Survival: 57; Death: 39

**** Median value: Survival: 1.32; Death: 2.0

Table 4 – Multivariable relationship between drug resistance and outcome

Outcome	Characteristics in model	Variable	N	OR	95% CI	P-value
Unfavourable outcome	Age		122			0.013
	DST	HMR	122	0.22	0.03-1.87	0.17
		RMR	122*	-	-	-
		MDR	122	12.4	1.17-132.3	0.037
Mortality	HIV status		88	6.17	0.92-41.3	0.061
	DST	HMR	88**	-	-	-
		RMR	88*	-	-	-
		MDR	88	63.9	4.84-843.2	0.002

OR = odds ratio; CI = confidence interval; DST = drug susceptibility test ; HMR = isoniazid monoresistant; RMR = rifampin monoresistant; MDR = multidrug resistant.
Unfavourable outcome – death, quadriparesis, severe cognitive handicap or blindness
* Perfectly predicts failure in this model so dropped from analysis
** Perfectly predicts success in this model so dropped from analysis

was less significant following adjustment for drug resistance (adjusted OR 6.17, 95%CI 0.92-41.3; p=0.061).

DISCUSSION

Children with TBM in the WCP of South Africa are young, generally present with advanced disease and, if they survive, are usually left with some form of disability. Rates of drug resistance are relatively low but this study has demonstrated that the time from first symptoms of TBM to the child being given appropriate, effective treatment is longer when the child's isolate is resistant to rifampin and/or isoniazid. Young age is associated with a poor outcome. In this study, Beijing strain was not associated with drug resistance and there was no association between Beijing strain and either poor outcome or death. MDR-TB, however, was strongly associated with both unfavourable outcome and death, even after adjusted analysis.

A study by Thwaites and colleagues demonstrated that adults with TBM were much more likely to die if infected with an organism resistant to both isoniazid and rifampin but had no increased risk if resistant to isoniazid alone and/or streptomycin [12]. Other work by the same group demonstrated that HIV infection in adults does not change the clinical presentation of TBM but does influence outcome [32]. A case series of adults from KwaZulu-Natal demonstrated that MDR-TBM was often associated with poor outcome [11] and a series from Durban described eight children with MDR-TBM, of whom seven died [33]. Caws and colleagues demonstrated a relationship between Beijing strain and both HIV infection and drug resistance in adults with

TBM [20]. However, Maree and colleagues found, as with our study, no association between strain type and either presentation or outcome in an investigation of children with TBM [17]. Other studies have demonstrated a relationship between strain type and disease phenotype in children [34] and in adults [15, 35] and a number of investigations have demonstrated that strain type, and the Beijing strain specifically, is associated with drug resistance [18, 19, 36, 37].

The association between low CSF lymphocyte count and poor outcome in TBM has been demonstrated in other studies [15, 38]. Previous investigations have shown a relationship between different strains and CSF lymphocyte count which we did not demonstrate. The inflammatory response to TBM is the cause of some of the pathology but it is clear from these and previous data that a failure to mount a lymphocyte response is associated with poor outcome.

In the WCP, children with TBM are treated with rifampin, isoniazid, pyrazinamide and ethionamide [39]. This is in contrast to the WHO guidelines which previously recommended rifampin, isoniazid, pyrazinamide and streptomycin for two months followed by isoniazid and rifampin for four months [40] but now recommends rifampin, isoniazid, pyrazinamide and ethambutol for two months followed by rifampin and isoniazid for ten months [41]. Isoniazid, pyrazinamide and ethionamide penetrate into the CSF well, rifampin adequately and ethambutol and streptomycin poorly [42]. In addition, a high proportion of MDR-TB cases have evidence of resistance to ethambutol and pyrazinamide, [43] implying that if a strain is MDR, ethambutol and pyrazinamide should not be assumed to be effective. One final factor that needs to be considered is the genotypic basis of drug resistance which is complicated by cross-resistance and co-resistance [44]. Resistance to isoniazid is usually caused by mutations in either the katG gene or the inhA promoter region. KatG mutations are usually associated with total resistance to isoniazid but if the mycobacteria posess an inhA promoter region mutation, this usually results in low-level isoniazid resistance which can be overcome by giving isoniazid at a higher dose (15-20mg/kg) [45, 46]. InhA promoter region mutations, however, usually result in ethionamide resistance. One explanation for the good outcomes in our study for children with HMR-TB might be that until the diagnosis was made and appropriate treatment started, they received a number of effective drugs with good CSF penetration. Using either the old or the new WHO guidelines this would not have been the case.

The majority (63%) of children presenting with TBM had an identified TB source case but few (15%) had been given preventive treatment. In addition to identifying a source case it is vital to determine the DST pattern of that source case to start appropriate preventive treatment or, if disease develops, disease treatment for the child. Although four of the five children with MDR-TB had been given some kind of

prior treatment, none had been treated appropriately. As children in contact with MDR-TB have been previously demonstrated to develop TB on isoniazid preventive treatment, [47] the correct preventive treatment for child contacts of MDR-TB remains unclear [48]. Although it is important to strive to obtain a microbiological diagnosis from the child, in reality only a small proportion of children with TBM have microbiological confirmation with DST. Most children are treated presumptively and unless a source case is identified, *M. tuberculosis* cultured and DST performed, cases of drug-resistant TBM in children will be missed. Where this is HMR-TBM, it is possible that the current local regimen will adequately treat the disease; however in the context of MDR-TBM outcome is poor unless appropriate second-line treatment is initiated rapidly. Of note, although over 85% of children had evidence of Bacillus Calmette-Guérin (BCG) vaccination, TBM still occurred. The protective efficacy of BCG remains debated and the need for effective vaccines is a pressing priority. Only 80% of children were tested for HIV, despite prolonged hospitalisation for a condition known to be associated with HIV infection. All children suspected of TBM should be tested for HIV, especially in a region with high HIV prevalence.

This study is retrospective and the data analysed is reliant on collection from routine sources such as case notes and laboratory records. As there were relatively few cases that had drug resistance, statistical analysis may not have revealed associations that may have been evident if a larger proportion of the cases had been drug-resistant. The children in our study may not be representative of all children with TBM. First, the study was carried out in a hospital which may have a more severe disease phenotype than those managed in the community. Second, as a positive mycobacterial result was required for inclusion in the study it is possible that the children had more advanced disease than is typical. A further limitation may have been that survival bias occurred with those presenting to TCH having a greater chance of both survival and drug resistance being diagnosed. Finally, we only recorded the outcome at the end of therapy. Longer follow up would have been desirable.

CONCLUSIONS

We have found a relationship between *M. tuberculosis* DST and clinical outcome in children with TBM, whereas no relationship between strain type and outcome or strain type and DST was evident. Drug-resistant TBM in children is associated with delay in initiation of appropriate therapy and MDR-TBM is associated with poor outcome. We did not, however, detect any difference in outcome between those with drug-susceptible TBM and those with HMR-TBM. One explanation may be the local treatment regimen employed in the WCP of South Africa. Further investigation into the use of this regimen, ideally by randomised, controlled trial, is indicated.

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Part III General discussion and summary

9 General discussion with overview of the studies and future perspectives

"The challenges posed by M. tuberculosis infection, through its interaction with the immune system and its mechanisms for evasion, require many more breakthroughs from basic science research if we are to make a significant impact on the worldwide tuberculosis problem." [1]

Annually, more than 6000 tuberculosis (TB)-related papers are published, but efforts to control TB are still hindered by gaps that exist in the diagnosis, prevention and treatment of this disease. Studies of the host immune response to TBM improve our understanding of the pathophysiology of this devastating disease and might direct future strategies to fill these gaps. Biomarkers are already well advanced for the diagnosis and prognosis of cancer, metabolic disorders and diabetes, and are gaining increasing importance in infectious diseases [1-4]. In this thesis, biomarker patterns on the level of proteins (i.e. proteomics) in the CSF and serum of children with signs and symptoms that are suggestive of meningitis were studied, and a unique and highly specific biomarker pattern in the CSF of patients with TBM was identified via the use of specific classification algorithms such as unsupervised hierarchical clustering analysis and principal component analysis (Chapter 2). Previous proteomic studies have identified biomarkers for TB in serum, that are capable of distinguishing pulmonary from extra-pulmonary TB and other infectious diseases [5, 6]. Other studies have revealed differential changes in smear-positive and -negative TB patients and controls [7]. Despite the numerous potential proteomic biosignatures that have been published over the years, the validation of these markers in clinical settings has proven challenging, [8] and between-study inconsistencies in the identified biomarkers are often observed [9].

Other methods for identifying disease-specific biosignatures include the study of the complete set of RNA transcripts at a specific time (i.e. 'transcriptomics') or the study of the sum of the small molecules that are involved in the metabolism of an organism (i.e. 'metabolomics' or 'metabolic profiling'). Currently we explore both techniques in our cohort of children with suspected meningitis to identify other potential biomarkers for future diagnostic and therapeutic purposes. Ultimately, predictions based on combined metabolite, transcript and protein signatures, will potentially result in more robust predictions and allow coherent biological exploration of the host response. However, this so-called multiplatform approach is currently not yet in widespread use.

In this general discussion, the results of the studies outlined in the previous chapters will be further discussed in the context of the three TB control pillars: diagnosis, prevention and treatment with specific attention to the host immune response and vitamin D.

DIAGNOSIS

Although TB is the second leading cause of death from an infectious disease globally, [10] and TBM is the most common cause of paediatric bacterial meningitis in the Western Cape, [11] there are still no available rapid, cheap and accurate diagnostic tests [5]. Early treatment initiation is important to avoid associated morbidity and mortality in TBM, which emphasises the importance of early diagnosis. Many attempts have been made to develop simplified, mostly antigen-detection tests for TB, but the diagnostic powers of these tests remain poor [12]. Microbiological confirmation of TBM is difficult and poses an even greater challenge in young children due to the paucibacillary nature of the disease and the low CSF volumes available for diagnostic analysis [13]. Direct microscopy for acid-fast bacilli in the CSF is fast but has very low sensitivity (<20%), [14] whereas mycobacterial culture has a slightly improved sensitivity, may take up to six weeks and rarely influences clinical management [15–17].

Nucleic acid amplification test

Several commercially available nucleic acid amplification tests (NAATs) have been developed for the rapid diagnosis of TBM. A systematic review and meta-analysis of the diagnostic accuracies of the newer commercial NAATs was performed by our research group and revealed that commercial NAATs exhibit good specificity (0.98) and a sensitivity of 0.64 in adults [18]. In the study presented in Chapter 7, we prospectively assessed the diagnostic accuracies of two commercial NAATs (Genotype MTBDR*plus*® and Xpert MTB/RIF®) on CSF collected from paediatric meningitis suspects. The combination of the two NAATs gave the best diagnostic performance with a combined sensitivity of 48% and a specificity of 98%. Techniques for optimising the concentration of microorganisms per volume CSF will improve test sensitivities and should be further evaluated. Although challenging in a paediatric population, it has been suggested that at least 6 ml of CSF should be collected and concentrated to improve the diagnostic yield [19]. At the beginning of treatment, some patients suffer a so-called 'paradoxical response', that is presumably an immunological reaction to the burst of mycobacterial contents that are released at the initiation of treatment [20, 21]. Enhanced release of mycobacterial DNA is therefore expected in the first few days after treatment initiation. One can speculate that repeated testing several days after treatment initiation might result in improved test sensitivity. As delay in initiation of appropriate treatment with second-line drugs in case of multidrug resistant (MDR)-TBM is associated with poor outcome (Chapter 8), the major advantage of the current NAATs compared to mycobacterial culture is the availability of test results within several hours and provides direct insight in drugs sensitivity. Although,

the sensitivities of the available commercial NAATs are still suboptimal in childhood TBM, results are promising.

Lipoarabinomannan antigen-detection assay

Among the most promising antigen-detection assays for the diagnosis of TB is an assay that is based on the detection of lipoarabinomannan (LAM), a *Mycobacterium*-specific lipopolysaccharide of the bacillus cell wall [22]. Intriguingly, urinary lipoarabinomannan detection seems to be of little diagnostic value in the diagnosis of TBM in a paediatric population of meningitis suspects (Chapter 6). Evaluation of the diagnostic accuracy of a commercial LAM ELISA test on CSF of 52 children diagnosed with TBM revealed the same disappointing result (data not shown in this thesis). None of the patients tested positive for LAM. LAM is released from metabolically active and disintegrating mycobacteria into the bloodstream [23] and can, when unbound, pass freely through the glomerular membrane. However, LAM can also bind to antibodies to form large immune complexes with limited capacities to pass through the glomerular membrane [24]. Because of their immunocompromised states, patients with HIV have higher mycobacterial loads [25] and decreased capacities for immune complex formation, [26] both of which result in increased levels of urinary LAM [27]. Given the inverse relationship between $CD4^+$ T-cell count and LAM ELISA sensitivity, [2] the low number of HIV-infected children with TBM in the study cohort presented in this thesis likely negatively influenced the diagnostic accuracy of the LAM ELISA test.

Point-of-care test based on biomarkers in CSF

Rapid, cheap and accurate diagnostic tests will improve diagnostic decision-making for patients with suspected TBM and decrease healthcare costs by preventing over-treatment of non-TBM patients. Compared to the current standard diagnostic tests for childhood TBM, the diagnostic biomarker based prediction model outlined in Chapter 2 is promising, although further refinement of the diagnostic model and validation in an independent external cohort are needed. Novel techniques such as the 'no-wash' (NW) assay platform developed by Becton, Dickinson and Company (BD), might be valuable for the development of a point-of-care test for TBM in the future. This NW-platform enables multiplexed detection of proteins in a broad range of samples and utilises a novel detection system that is based on Surface Enhanced Raman Spectroscopy (SERS)-active nanoparticles.

PREVENTION

Over the past 5 years, an alarming increase in the number of patients with MDR-TB and extensively drug-resistant TB has been noted [28] along with related adverse outcome in case of TBM (Chapter 8). Unfortunately, the development of new drugs does not keep pace with the development of drug resistance. Therefore, the development of a universally protective tuberculosis vaccine is desperately needed. The BCG vaccine, which was developed nearly a century ago and protects against serious forms of disseminated disease, is the only TB vaccine that is currently in use. In the last decade, several promising vaccine candidates for tuberculosis have been developed, but these candidates remain at different stages of the clinical trial pipeline [28]. The identification of correlates of protective host immune responses would aid the development of superior disease prevention strategies [28]. Intriguingly in this context is the role of vitamin D in the host immune response to tuberculosis. As vitamin D deficiency has been associated with an increased risk of tuberculosis [30], biological intervention strategies with vitamin D or vitamin D-related antimicrobial biomarkers might be beneficial.

The role of vitamin D

Currently, the best explanation for low vitamin D levels in patients with active TB is that fall in serum 25(OH)D concentration activates latent disease [31]. Because vitamin D is primarily derived from cutaneous photosynthesis in the presence of ultraviolet-B (UVB) radiation, its production reaches its minimum during winter months in countries at higher latitudes. Non-Western populations who migrate to these countries are known to be at increased risk for vitamin D deficiency [32] and consequently for *M. tuberculosis* disease activation [33]. Contributory factors to this increased risk are a dark skin type, less time spent outdoors, low socioeconomic status, the wearing of body-covering clothes, and a diet low in fish and dairy products [32]. In Chapter 5, we evaluated serum vitamin D levels of a paediatric population of native Dutch and first- and second-generation non-Western immigrants (Chapter 5) and found differences in serum vitamin D levels between these 3 groups; the lowest vitamin D levels were observed in the first-generation non-Western immigrants. Acculturation, i.e. ''the dual process of cultural and psychological change that takes place as a result of contact between two or more cultural groups and their individual members'', [34] might contribute to this phenomenon. There is increasing evidence that acculturation is associated with significant changes in the obesogenic behaviours of populations of immigrants that have moved from low- and medium-income countries to high-income countries [35] but the impact on vitamin D levels over time have not been well described. Further research in this field is essential to improve

our understanding of the contribution factors that lead to these differences in serum vitamin D and will subsequently lead to improvements in TB controlling strategies for immigrants migrating to temperate regions.

The hypothesis that fall in serum 25(OH)D concentration activates latent disease [31] is reinforced by the seasonal variation in the incidence of diseases caused by *M. tuberculosis*; [36, 37] the highest incidence rates of TBM are observed during late winter and early spring (Chapter 3; [38]). In this context, we studied the association between seasonal variation in the incidence rate of TBM and sunshine hours prior to disease manifestation in a retrospective cohort of patients with TBM (Chapter 3) and found a significant association between the incidence rate of TBM and sunshine hours 3 months earlier. In Chapter 4, we prospectively explored the role of vitamin D in the pathophysiology of TBM and found an evident association between TBM and low serum levels of vitamin D in a population with suspected meningitis.

Vitamin D-mediated antimicrobial response in TBM

Since Rook *et al*. [39] demonstrated that 1,25-dihydroxyvitamin D3 (1,25D) inhibits *M. tuberculosis* replication, the evidence that vitamin D contributes to the host innate immune response of patients with TB by maintaining the localised production of cathelicidin LL-37 following the activation of monocytes by TLR ligands has grown [40, 41]. The activation of TLR 2/1 results in the induction of key genes in the vitamin D antimicrobial pathway, including the vitamin D receptor (VDR) and 25-hydroxyvitamin D3-1a-hydroxylase, which converts 25(OH)D into the active form (1,25D) [40]. Once converted, 1,25D binds to the intracellular vitamin D receptor (VDR). This nuclear receptor is found in several cells that are involved in the human immune system, including microglial cells [42]. Under conditions in which 25(OH)D is present at sufficient levels, the activation of monocytes by *M. tuberculosis* through the TLR signalling pathway can cause VDR-dependent production of pro- and anti-inflammatory cytokines and the expression of antimicrobial peptides, such as cathelicidin LL-37 [43]. Cathelicidin LL-37 represents one of the antimicrobial components of macrophages and is cleaved from the C-terminal portion of the Human cationic antimicrobial protein (*hCAP-18*) by proteinase 3. In TB, the induction of cathelicidin LL-37 in macrophages promotes the destruction of intracellular *M. tuberculosis* [40] and directs the differentiation of macrophages towards a pro-inflammatory phenotype [44]. High levels of cathelicidin LL-37 are present in CSF of patients with TBM compared to CSF of patients with other types of meningitis, (Chapters 3 and 4) and cathelicidin LL-37 levels are positive correlated with other vitamin D-related biomarkers (Chapter 4). Cathelicidin LL-37 plays a central role in the vitamin D-mediated human antimicrobial response and can therefore be used as valuable diagnostic marker to differentiate between TBM and other types of meningitis as described earlier.

Ex vivo, low levels of 25(OH)D (< 75 nmol/L) are associated with decreased *hCAP-18* mRNA expression by monocytes following immune challenges by TLR ligands, with improvement of the immune response after vitamin D supplementation [41]. Intriguingly, this phenomenon was not observed in patients with TBM in vivo (Chapter 4). Given that cathelicidin LL-37 production in monocytes seems to be dependent on both TLR ligands and 25(OH)D, the high levels of CSF cathelicidin LL-37 in a relatively low 25(OH)D level environment of patients with TBM is suggestive of a vigorous immune stimulation by *M. tuberculosis*-specific TLR ligands that might disturb the correlation between serum 25(OH)D and CSF cathelicidin LL-37 in TBM patients. Although CSF features are frequently used in diagnostic prediction rules to differentiate between different types of meningitis [45, 19], they poorly predict clinical outcome in TBM [16]. As many sequelae of TBM result from an immunologically directed inflammatory response to the infection [46] one would expect a stronger correlation between CSF features and clinical outcome. It is possible that inflammatory products, such as cathelicidin LL-37, that are measured in CSF of patients with TBM, might not fully reflect the localised inflammatory response in the brain tissue and might therefore contribute to the lack of correlation between serum 25(OH)D and CSF cathelicidin LL-37 concentrations.

TREATMENT

TBM treatment includes four drugs, which were developed more than 40 years ago, and only prevents death or severe disability in fewer than half of patients [47]. Emerging resistance to current drugs necessitates the development of newer and better drugs. As previously stated, host-directed immunotherapeutic approaches to induce or expand clinically relevant anti-*M. tuberculosis* immune responses might be helpful [29]. Immunotherapeutic approaches often focus on cellular immune (adaptive or innate) responses, but other viable options include components that are involved in the vitamin D-mediated human antimicrobial pathway. The exposure of patients with TB to sunlight and vitamin D2 supplementation to treat TB were common practices in the pre-antibiotic era [48, 49]. Surprisingly, only a few clinical trials have addressed the effects of vitamin D supplementation during treatment of active TB on clinical outcomes with conflicting results [50–54]. Sputum culture conversion seems to only be accelerated by vitamin D3 adjunctive therapy in patients with the tt genotype of the TaqI VDR polymorphism [52].

By taking advantage of the experience that has been gained in the successful combat of other infectious diseases, Robert Koch attempted to fight against the tuberculosis epidemic in his time [55]. Currently, pathway analysis helps us to find

overlaps with pathogenic pathways of other diseases and improves our understanding of the pathophysiology of TBM. In TBM, there are overlaps with the pathogenic pathway of multiple sclerosis (MS), the VDR/RXR activation pathway, and CCR5 signalling in macrophages (Chapter 2). Influencing the trafficking of immune cells and their transfer to lesion sites are potential targets for the treatment of MS [56]. The upregulation of MS-related signalling in TBM suggests that similar treatment modalities might affect the sequelae of TBM. The involvement of components of the VDR/RXR pathway supports a putative role for vitamin D in the pathophysiology of TBM and is reminiscent of autoimmune diseases, such as MS.

FUTURE PERSPECTIVES

Translating knowledge gained from research is essential for successful implementation with the ultimate goal of improving health care. Building on the identified biomarkers, the development of an accurate point-of-care test for the early diagnosis of TBM in low-income and recourse-poor settings can make that required transition to practical implementation. Following development and validation, the use of this test might successfully be rolled out and scaled-up not only in other hospitals of the Western Cape or South Africa but other resource-poor settings that are affected by TBM across the world to improve health outcomes globally. Only 28 host markers were evaluated in this thesis, and numerous additional components are evaluable with novel techniques such as the multiplatform approach.

It has previously been demonstrated that pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α), interleukin (IL)-6, IL-1β and interferon [IFN]-γ, exhibit seasonal variations in healthy subjects and are related to serum vitamin D levels [57]. Disease activation from a state of latency in TBM is thought to depend on the balance between pro- and anti-inflammatory cytokines, [46] and therefore seasonal variation in serum 25(OH)D levels and those of related biomarkers might cause disease activation. Whether serum vitamin D levels influence the inflammatory response in active *M. tuberculosis* disease is unclear, although preliminary data from our group suggest vitamin D dependence in TBM patients (Figure 1). Further research on vitamin D-dependent biomarker profiles during and prior to active disease is warranted. TBM models, such as the 'in silico model' of El-Kebir *et al*. [58] or animal models such as the TBM zebrafish model [59] and TBM mouse model [60] that have been developed by our research group might be useful for this purpose.

Host-directed immunotherapeutic approaches inducing or expanding clinically relevant anti-*M. tuberculosis* immune responses need to be further evaluated. As

genetic differences in the host, such as VDR polymorphisms, influence the quality of the immune response to *M. tuberculosis*, advanced knowledge of these genetic variants might guide individual therapeutic approaches in the future. The role of antimicrobial peptides in the clearance of intracellular *M. tuberculosis* seems to be important and might benefit future therapeutic strategies as well.

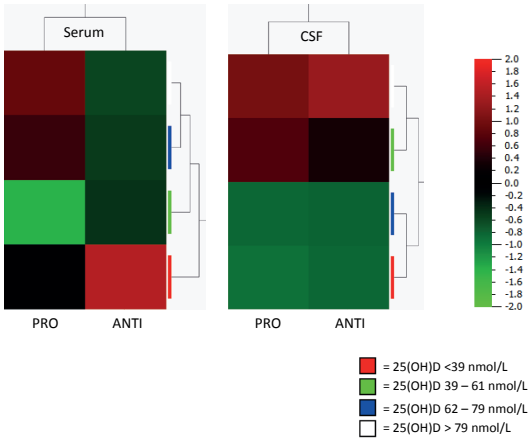

Figure 1
Two-dimensional unsupervised hierarchical clustering (UHC) of 25-hydroxyvitamin D-dependent subgroups of tuberculous meningitis (TBM) patients and their pro- and anti-inflammatory cytokine expression profiles (Pro-inflammatory cytokines: IL-1β, IL-2, IL-6, IL-12 (p70), TNF-α and IFN-γ; anti-inflammatory cytokines: IL-1ra, IL-4, IL-5, IL-10 and IL-13). Prior to UHC analysis, the 48 TBM cases were ranked in ascending order and tiled in four subgroups (n=12) based on serum 25(OH)D level. Grouped medians were taken to illustrate differences in pro- and anti-inflammatory cytokines between the four TBM subgroups. The normalised values for each cytokine group are depicted according to the colour scale, in which red and green represent expression above and below the median, respectively. The dendogram (right) shows the proximity between the four vitamin D subgroups. In serum clear differences in pro- and anti-inflammatory cytokine expression between the four TBM subgroups are present; a shift from a relative abundance of anti-inflammatory cytokines in TBM patients with low serum vitamin D levels to a pro-inflammatory cytokine expression profile of TBM patients with high serum vitamin D levels can be observed. This shift was not observed in CSF samples.

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10 Summary & Nederlandse samenvatting

SUMMARY

Worldwide, tuberculosis (TB) remains a major health problem with efforts to control TB still hindered by gaps in knowledge that exist in diagnosis, prevention and treatment. After exposure, human infection with *Mycobacterium (M.) tuberculosis* can progress to active disease, be contained as latent infection, or be eradicated by the host immune response. Central nervous system involvement occurs in ~1% of all cases of TB, among which cases of tuberculous meningitis (TBM) are the most severe manifestation and frequently occur during early childhood. Although the clinical presentation and histo-pathological mechanisms of TBM are well-defined over the last decades, the cellular and molecular mechanisms are still poorly understood. A successful host response to an invading pathogen requires precise co-ordination of the components of the immune system. The studies described in this thesis aim to better understand the host immune response to TBM in order to improve future diagnostic, preventive and therapeutic strategies.

PART I – In the first part of this thesis the host immune response to TBM and the role of vitamin D is investigated. In Chapter 2 biomarker patterns in cerebrospinal fluid (CSF) and serum of children resident in a TB endemic area, with signs and symptoms suggestive of meningitis, are evaluated by using multiple statistical analyses. Unsupervised hierarchical clustering analysis revealed a disease-specific pattern of biomarkers for TBM relative to other forms of meningitis. Pathway analysis indicated that biomarkers involved in TBM pathogenesis resembled those involved in multiple sclerosis, and that involved in vitamin D receptor / retinoid X receptor activation were over-represented in TBM compared to other forms of meningitis. With these results, a promising CSF biomarker-based diagnostic prediction model for childhood TBM has been developed, based on three biomarkers: interleukin-13, vascular endothelial growth factor, and cathelicidin LL-37. The results of this study highlight the potential of bio-signatures in the host's CSF for diagnostic applications and for improving our understanding of the pathogenesis of TBM to discover strategies to prevent immune-pathological sequelae.

The incidence rate of TBM varies with season, and serum vitamin D levels, which are dependent on sunlight, may play a role. Chapter 3 describes the association found between the seasonal variation in the incidence rate of TBM and sunshine hours prior to disease manifestation. This association supports the hypothesis that vitamin D has a role in the pathophysiology of TBM. In Chapter 4 a prospective study is described in children with suspected meningitis in which serum 25-hydroxyvitamin D (25(OH)D) levels and CSF concentrations of cathelicidin LL-37 and five other vitamin-D related biomarkers were investigated. Low serum 25(OH)D levels were

found to be associated with a TBM diagnosis, while CSF cathelicidin LL-37 concentrations are relatively high in TBM patients when compared to non-TBM patients. Furthermore, significant correlations were found between cathelicidin LL-37 and concentrations of interleukin (IL)-13, Interferon-γ (IFN-γ), Regulated on activation normal T cell expressed and secreted (RANTES) and IFN-γ-induced protein-10 in CSF. All together, these findings stress the role of vitamin D in the pathophysiology of TBM.

Currently, the best explanation for low vitamin D levels in patients with active TB is that a fall in serum 25(OH)D concentration activates latent disease. As vitamin D is mainly derived from cutaneous photosynthesis in the presence of Ultraviolet-B radiation, its production declines to a minimum during winter months in countries at higher latitudes. Immigrants to these countries are known to be at increased risk for vitamin D deficiency and consequently for *M. tuberculosis* disease activation. In the last chapter of part one, the association between migration status and vitamin D deficiency in a Dutch paediatric population is studied (Chapter 5). Differences in serum vitamin D levels were found between native Dutch and non-Western immigrants, with lowest vitamin D levels in first-generation non-Western immigrants.

PART II – The second part of this thesis focuses on improving the early diagnosis of childhood TBM and the impact of drug resistance on clinical outcome. The main difficulty with diagnosis of TBM in children is its paucibacillary nature. Among the most promising antigen-detection assays for diagnosing TB is an assay based on the detection of lipoarabinomannan (LAM), a *Mycobacterium*-specific lipopolysaccharide of the bacillus cell wall. Chapter 6 describes the diagnostic accuracy of a commercial urine LAM antigen-detection assay. In contrast with results of studies done in adults, urinary LAM detection appeared to be of little diagnostic value for the diagnosis of TBM in a paediatric population of meningitis suspects (sensitivity 4.8% and specificity 93.1%). Given the inverse relationship between $CD4^+$ T-cell count and urinary LAM sensitivity, the low number of HIV-infected TBM patients in our study cohort could have contributed to the low sensitivity observed. In the study presented in Chapter 7 we prospectively assessed the diagnostic accuracies of two commercial nucleic acid amplification tests (NAATs, Genotype MTBDR*plus*® and Xpert MTB/RIF®) on CSF collected from paediatric meningitis suspects. Using a pre-defined TBM case-definition as reference standard, sensitivities and specificities were 32% and 98% for MTBDR*plus*®, and 25% and 100% for Xpert MTB/RIF®. The combination of the two NAATs gave the best diagnostic performance with a combined sensitivity of 48% and a specificity of 98%. Given the low sensitivities of CSF microscopy and liquid culture, the improved diagnostic performance achieved with commercial NAATs is encouraging.

During the past 5 years, an alarming increase in the number of patients with multidrug-resistant (MDR)-TB and extensively drug-resistant TB has been noted. In the last chapter of Part II, the impact of drug resistance on clinical outcome in children with TBM is discussed (Chapter 8). MDR-TBM in children seems to have poor clinical outcome and is associated with death. No difference in the outcomes between children with isoniazid mono-resistant TBM and those with drug-susceptible TBM were found. A probable explanation for this finding could be due to the anticipation of isoniazid mono-resistance by the local TBM treatment regimen used in this study.

NEDERLANDSE SAMENVATTING

Tuberculose (TB) is wereldwijd nog steeds een belangrijk gezondheidsprobleem. Onvoldoende kennis over diagnostiek, preventie en behandeling beperkt de mogelijkheden om deze ziekte goed te controleren. Na blootstelling kan besmetting met *Mycobacterium (M.) tuberculosis* resulteren in actieve ziekte, latente ziekte of eliminatie van het micro-organisme, waarbij het menselijk afweersysteem een belangrijke rol speelt. Bij ongeveer 1% van alle patiënten met een actieve vorm van TB is het centraal zenuwstelsel betrokken. Daarvan is hersenvliesontsteking door *M. tuberculosis*, ook wel tuberculeuze meningitis (TBM) genoemd, de meest ernstige vorm. Deze komt frequent voor op de vroege kinderleeftijd. Alhoewel gedurende de laatste decennia de klinische symptomen en het histopathologische mechanisme van TBM goed zijn beschreven, zijn de onderliggende cellulaire en moleculaire mechanismen minder goed onderzocht. Een succesvolle menselijke afweerreactie op een binnendringend micro-organisme vereist precieze coördinatie van de componenten van het menselijk afweersysteem. De studies die in dit proefschrift worden beschreven hebben als doel om de menselijke afweerreactie optredend bij TBM beter te begrijpen om uiteindelijk de bestaande diagnostische, preventieve en therapeutische strategieën te kunnen verbeteren.

DEEL I – In het eerste deel van dit proefschrift wordt de menselijke afweerreactie optredend bij TBM en de rol die vitamine D hierbij speelt onderzocht. In hoofdstuk 2 worden met behulp van verschillende statistische technieken ziekte-specifieke ontstekingsmoleculen bestudeerd die gevonden zijn in het bloed en hersenvocht van kinderen met verdenking op hersenvliesontsteking die woonachtig zijn in een TB endemisch gebied. 'Unsupervised hierarchical clustering' (UHC) analyse laat een voor TBM specifiek patroon van ontstekingsmoleculen zien wanneer dit vergeleken wordt met andere vormen van meningitis. Dit patroon van TBM specifieke ontstekingsmoleculen heeft overeenkomsten met die van multiple sclerose en met moleculen betrokken bij de vitamine D receptor / retinoid X receptor activatie. Aan de hand van deze bevindingen is een veelbelovend diagnostisch voorspelmodel voor TBM ontwikkeld, gebaseerd op 3 ontstekingsmoleculen in het hersenvocht: interleukine-13, 'vascular endothelial growth factor' en cathelicidin LL-37. De resultaten gepresenteerd in hoofdstuk 2 benadrukken het belang van het bestuderen van patronen van ontstekingsmoleculen in het menselijk hersenvocht om onze kennis te verbeteren over het pathomechanisme van TBM en om toekomstige diagnostische, preventieve en therapeutische strategieën te kunnen ontwikkelen.

De incidentie van TBM varieert per seizoen. Vitamine D, dat gevormd wordt onder invloed van zonlicht en betrokken is bij het menselijk afweersysteem, speelt hierin

mogelijk een rol. Hoofdstuk 3 beschrijft de associatie die is gevonden tussen de seizoen variatie in de incidentie van TBM en het aantal zonuren voorafgaand aan de ziekte. Met het aantonen van deze associatie wordt de hypothese dat vitamine D een rol speelt in het optreden van TBM ondersteund. In Hoofdstuk 4 wordt een studie beschreven waarin bij kinderen met verdenking op hersenvliesontsteking is gekeken naar de relatie tussen het serum 25-hydroxyvitamine D (25(OH)D) gehalte en hersenvocht concentraties van cathelicidin LL-37 en vijf andere vitamine D gerelateerde ontstekingsmoleculen. Er wordt beschreven dat een laag serum 25(OH) D gehalte is geassocieerd met de diagnose TBM en dat de concentratie van cathelicidin LL-37 in het hersenvocht relatief hoog is van kinderen met TBM in vergelijking tot kinderen met andere vormen van meningitis. Tevens wordt er beschreven dat er significante correlaties bestaan tussen cathelicidin LL-37 en concentraties van interleukine (IL)-13, Interferon-γ (IFN-γ), 'Regulated on Activation Normal T cell Expressend and Secreted' (RANTES) en IFN-γ-induced protein-10 in het hersenvocht. Deze bevindingen benadrukken de rol van vitamine D in het pathomechanisme van TBM.

Op dit moment is de beste verklaring voor de associatie tussen een laag serum vitamine D en een actieve vorm van TB dat het dalen van de vitamine D serumconcentratie zorgt voor ziekte activatie. In de mens wordt vitamine D voornamelijk geproduceerd door fotosynthese in de huid onder invloed van Ultraviolet-B straling. In landen gelegen op een hoge breedtegraad is de hoeveelheid UVB in het zonlicht tijdens de wintermaanden zeer gering, dit leidt tot een daling van de serumconcentratie vitamine D. Van niet-Westerse populaties die naar deze landen gemigreerd zijn is bekend dat zij een verhoogd risico hebben op vitamine D deficiëntie vanwege onder andere hun donkere huidskleur. De consequentie is een verhoogd risico op ziekte activatie in het geval er sprake is van besmetting met *M. tuberculosis*. In het laatste hoofdstuk van deel 1 (Hoofdstuk 5) wordt de associatie tussen migratie status en vitamine D serumconcentratie onderzocht in een populatie kinderen woonachtig in Nederland. Verschillen in vitamine D serumconcentratie werden gevonden tussen autochtone Nederlandse kinderen en niet-Westerse immigranten, waarbij de laagste vitamine D concentraties werden gevonden in kinderen behorende tot de eerste generatie niet-Westerse immigranten.

Deel II – Het tweede deel van dit proefschrift richt zich op de verbetering van diagnostische mogelijkheden voor het stellen van de diagnose TBM op de kinderleeftijd en de invloed van medicatie-resistentie op de klinische uitkomst. Het aantonen van de ziekte TBM op de kinderleeftijd wordt bemoeilijkt door de lage sensitiviteit van de meeste diagnostische tests als gevolg van de lage bacteriële load in het hersenvocht. Een veelbelovende nieuwe test voor het stellen van de diagnose TB is gebaseerd op het detecteren van het antigeen lipoarrabinomannan (LAM), een *Mycobacterium-*

specifiek lipopolysaccharide van de bacteriele celwand. In Hoofdstuk 6 wordt een commerciële urine LAM antigeen-detectie test geëvalueerd. In tegenstelling tot de resultaten van studies die gedaan zijn bij volwassenen blijkt de test van weinig diagnostische betekenis te zijn voor de diagnose TBM in een populatie kinderen met TBM (sensitiviteit 4.8% en specificiteit 93.1%). Gegeven de omgekeerde relatie tussen $CD4^+$ T-cel aantallen en de sensitiviteit van LAM antigeen-detectie in de urine, heeft het lage aantal HIV positieve kinderen in het cohort beschreven in Hoofdstuk 6 mogelijk bijgedragen aan de lage waargenomen sensitiviteit. De studie beschreven in Hoofdstuk 7 heeft gekeken naar de diagnostische waarde van twee commerciële 'Nucleic acid amplification tests' (NAATs, Genotype MTBDR*plus*® and Xpert MTB/RIF®**),** getest op hersenvocht van kinderen die verdacht werden van hersenvliesontsteking. Door een op consensus gebaseerde klinische ziekte-definitie te gebruiken als gouden standaard voor de diagnose TBM, werd een sensitiviteit en specificiteit van 32% en 98% voor MTBDR*plus*®, en 25% en 100% voor Xpert MTB/RIF® gevonden. Door de twee NAATs te combineren werd het beste diagnostische resultaat verkregen met een gecombineerde sensitiviteit van 48% en een specificiteit van 98%. Gegeven de lage sensitiviteit van microscopisch onderzoek en bacteriële kweek van het hersenvocht, zijn de resultaten die bereikt zijn met de commerciële NAATs bemoedigend.

Gedurende de laatste vijf jaar is er sprake van een verontrustende stijging van het aantal patiënten met 'multidrug'-resistente (MDR)-TB en 'extensively drug' resistente (XDR)-TB. In het laatste hoofdstuk van dit deel wordt de invloed van medicatie resistentie op de klinische uitkomst van kinderen met TBM bestudeerd (Hoofdstuk 8). Op basis van deze studie wordt geconcludeerd dat MDR-TBM bij kinderen leidt tot een slechtere klinische uitkomst en is geassocieerd met overlijden aan de ziekte. Er werden geen verschillen gevonden in klinische uitkomst tussen kinderen met een isoniazide mono-resistente vorm van TBM en kinderen met medicatie gevoelige TBM. Het behandelprotocol dat werd gebruikt in deze studie anticipeert op isoniazide mono-resistentie en verklaart mogelijk deze laatste bevinding.

Addendum

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Douwe Visser was born in 1980 in Soest, The Netherlands. In 1998 he graduated from high school and started his medical training at the Faculty of Medicine at the Vrije Universiteit in Amsterdam where he graduated cum laude as Doctor of Medicine (MD) in 2005. Next to his medical training, he studied for 2 years at the Sweelinck conservatorium, school of music in Amsterdam. After obtaining his medical degree Douwe worked as a resident in pediatric surgery and started his paediatric training-ship in 2006. During this training he worked at the VU University Medical Center and Sint Lucas Andreas Hospital in Amsterdam and started the research project described in this thesis. In 2011 Douwe was registered as Paediatrician and started his fellowship in Neonatology at the VU University Medical Center. Since November 2014 he works as Paediatric Consultant / Neonatologist at the VU University Medical Center. Douwe happily lives together with Sanne Hunfeld and their children Hanna, Toon & Luc and Nynke.

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- Solomons RS, Goussard P, Visser DH, Marais BJ, Gie RP, Schoeman JF, van Furth AM. Chest radiograph findings in childhood tuberculous meningitis. *Submitted*

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ABBREVIATIONS

1,25D	1,25-dihydroxyvitamin D3
25(OH)D	25-hydroxyvitamin D
ART	antiretroviral therapy
AUC	area under the curve
BCG	bacillus Calmette-Guérin
BCH	Brooklyn Chest Hospital
bFGF	eotaxin, basic fibroblast growth factor
BM	bacterial meningitis
BMI	body mass index
CAS	Central Asian lineage
CCR	CC chemokine receptor
CI	confidence interval
CNS	central nervous system
CR	chest radiograph
CSF	cerebrospinal fluid
CT	computerized tomography
CV	coefficient of variation
CXR	chest X-ray
DQ	development quotient
DS	drug susceptibility
DST	drug susceptibility testing
ELISA	enzyme-linked immunosorbent assay
FG	first generation
G-CSF	granulocyte colony-stimulating factor
GAW	global atmosphere watch
GCS	Glasgow coma score
GM-CSF	granulocyte–macrophage colony-stimulating factor
hCAP-18	human cationic antimicrobial protein
HIV	human immunodeficiency virus
HMR	isoniazid mono-resistant
IFN-γ	interferon-γ
IGRA	Interferon-Gamma Release Assay
IL	interleukin
IMR	isoniazid monoresistant
IP	IFN-γ-induced protein
IQR	interquartile range

IRR	incidence rate ratio
LAM	lipoarabinomannan
LAM	Latin American and Mediterranean family
LCC	Low Copy Number Clade
LPS	lipopolysaccharide
M.	Mycobacterium
MCP	monocyte chemotactic protein
MD	Doctor of Medicine
MDR	multidrug-resistant
MED	minimum erythema dose
MGIT	Mycobacterial Growth Indicator Tubes
MIP	macrophage inflammatory protein
MS	multiple sclerosis
NAAT	nucleic acid amplification test
ND	native Dutch
NPV	negative predictive value
NS	not significant
NW	no-wash
OD	optical density
PAMP	pathogen-associated molecular pattern
PCA	principal component analysis
PCR	polymerase chain reaction
PDGF	platelet derived growth factor BB
PPV	positive predictive value
PRR	pattern recognition receptor
RA	receptor antagonist
RANTES	regulated upon activation normal T-cell expressed and secreted
RMR	rifampin mono-resistant
ROC	receiver operating characteristic
RXR	retinoid X receptor
SD	Standard deviation
SERS	surface enhanced raman spectroscopy
SG	second generation
TB	tuberculosis
TBM	tuberculous meningitis
TCH	Tygerberg Children's Hospital
Th	T helper
TLR	Toll-like receptor
TNF-α	tumour necrosis factor-α

TST	tuberculin skin test
UHC	unsupervised hierarchical clustering
UVB	Ultraviolet-B
VDR	vitamin D receptor
VEGF	vascular endothelial growth factor
VM	viral meningitis
WCP	Western Cape Province
WHO	World Health Organization
XDR	extensively drug-resistant